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PRINCIPAL INVESTIGATOR: C. Guillermo Couto, DVM, Diplomate ACVIM

CONTRACTING ORGANIZATION: The Ohio State University
Columbus, OH 43210

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14. ABSTRACT

The purpose of this proposal is to provide insight into gene environment interactions. It leverages the simplified genetics and detailed records of the military working dog population. There are several critical aspects to meeting the aims of this proposal. 1) development of data driven selection criteria, 2) biological sampling of representative dogs, and 3) generation of mathematical methodologies capable of handling heterogenous data and statistical tests in consistent manner and providing clear and understandable results that are biologically valid. Here we provide a breakdown of the previous year's work and document our progress towards achieving the specific aims we proposed.

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Introduction

The purpose of this proposal is to provide insight into gene environment interactions. It leverages the simplified genetics and detailed records of the military working dog population. There are several critical aspects to meeting the aims of this proposal. 1) development of data driven selection criteria, 2) biological sampling of representative dogs, and 3) generation of mathematical methodologies capable of handling heterogenous data and statistical tests in consistent manner and providing clear and understandable results that are biologically valid. Here we provide a breakdown of the previous year's work and document our progress towards achieving the specific aims we proposed.

Body

Task 1- Regulatory Approval:

i) Cooperative Research And Development Agreements (CRADAs): Both the data and biological CRADAs between Nationwide Children's Hospital (NCHRI; Alvarez, Lead PI, home institution)/OSU (Huang and Couto, Partnering PI's) and DoD/USA were executed by 2013.

ii) Animal use approval (Institutional Animal Care and Use Committee, IACUC): The animal hospital at Lackland AFB received AAALAC accreditation that is mandatory for military IACUC approvals in 2012. In 2013, we submitted final revisions on our IACUC protocol for the collection of biological samples and Lackland veterinary approval was granted; and final Lackland AFB oversight approval was granted and those documents were submitted to DoD CDMRP grant administration. Currently, there is one final approval from ACURO pending (and expected, according to their original anticipated timeline, within ~1 month), at which time biological sample collection can be initiated.

Task 2- Data Capture of Veterinary Records: By having Ms. Michelle Perez, Veterinary Technician, embedded in the military dog health service at Lackland AFB, we have been acquiring clinical and associated data from military dogs. This was made possible by the execution CRADA's in 2013 (Task 1). The veterinary clinical cancer and medical records expertise was provided by Dr. Couto. We have been using that data in two parallel tracks. (i) In the first track, we have been using data forms to create advanced methods for capturing paper-based data and converting those to electronic data (which is classified as raw or manually confirmed to accurately represent the original) (using custom form versions of ABBYY software). That work was initiated in the technical sense before we had CRADA's in place to use it on real DoD military dog health records. In 2013, Mr. Terry Camerlengo and his subsequent replacement Mr. Jacob Aaronson (under supervision of Drs. Alvarez and Huang) worked with actual military dog health records (scanned by Vet. Tech. Ms. Perez at Lackland AFB) to create those custom electronic versions of paper forms. Specifically, they initiated the development of custom scanning and data capture from DoD military dog health record form 1829 (which are generated for each health visit, providing longitudinal data) and from AFIP/JPC pathology reports (which are generated for essentially all diagnostic cancer biopsies and sometimes for necropsy). That required significant efforts from ABBYY support and Research IT, NCHRI to implement. This effort is ongoing. If one or both final customized forms are successful in the near future, we will be able to scan any future records and automatically isolate each 1829 and pathology report. Importantly, we would also be able to scan the many prioritized full records scanned and archived in our database in "track ii". (ii) In the second track that was initiated in 2012 and is ongoing through 2013, we have used different indicators to prioritize individual dogs that are particularly important to our study and have begun scanning their complete records (except for some associated clinical test data that could not be scanned – e.g., EKG's on thin perforated paper (which would have risked their destruction in our portable automatic-feed scanner). We are mainly focused on dogs that have had cancer or most likely would have had it by now if they had high risk (according to age). We thus acquired a list of all Lackland AFB dog health records for which there are AFIP/JPC pathology reports. This was made possible by our primary military dog program contact, LTC Cyle Richard. He provided us that list, which he received from AFIP/JPC; in this way, we did not have to review thousands of records to identify those that contained pathology reports or cancer diagnoses. This in turn allowed us to examine DoD military dog puppy program dog (DoD bred dogs vs. purchased dogs) pedigrees for selection of affected and unaffected littermates or half siblings. From this analysis we identified a relatively small number of popular breeders that had many litters with different partners.

Task 3-Methodolgy Development:

Task 3 is advanced about as far as the data types we have acquired to date. Once final IACUC approval is granted (expected within the month) and we begin to acquire military dog samples after, we expect to be able to deploy the methodologies we have developed. Specifically, we have validated the principal new methods using data from previously-acquired Greyhound osteosarcoma case and control samples, and from data published by the LUPA Consortium (Vaysse A et al. 2011. Identification of genomic regions associated with phenotypic variation between dog breeds using selection mapping. PLoS Genet. 7(10):e1002316. PubMed PMID: 22022279).

In the first year's Annual Report, we included two manuscripts (Rybaczyk et al. and Rowell et al.) that used a new methodology we developed under the present program. Both those manuscripts were submitted for publication in leading genetics journals, and we have been addressing reviewers criticisms and advice. Throughout 2013, we continued to refine and validate those studies. Specifically, this work involves the invention of entirely novel techniques to conduct genomewide association analysis or GWAS (Balding 2006) and multidimensional statistical analysis: Intersection Union

Testing or IUT (Berger 1982; Berger 1997) combined with Bootstrapping (both well established, but the approach has never been used for these applications).

The original focus of these works was on development of the IUT. In the course of improving the methods to address reviewer comments during this reporting year, we determined that the integration of Bootstrapping with IUT is a major innovation and advantage (Fig. 1). The greatest concern about our manuscripts was that the IUT method does not generate conventional measures of statistical significance (p-values), despite the fact that the method empirically ranked IUT-"significant" hits correctly (according to detection of true positives in published datasets). [Notably, that is the major focus of applications of IUT to biology and high throughput gene expression data. Some have proposed solving it using Bayesian approaches, but after many years, no one has had success doing so.] By adding Bootstrapping upstream of IUT, we are able to give another type of measure of robustness of results – a confidence (vs. significance) measure (Bootstrap Confidence Value, BCV).

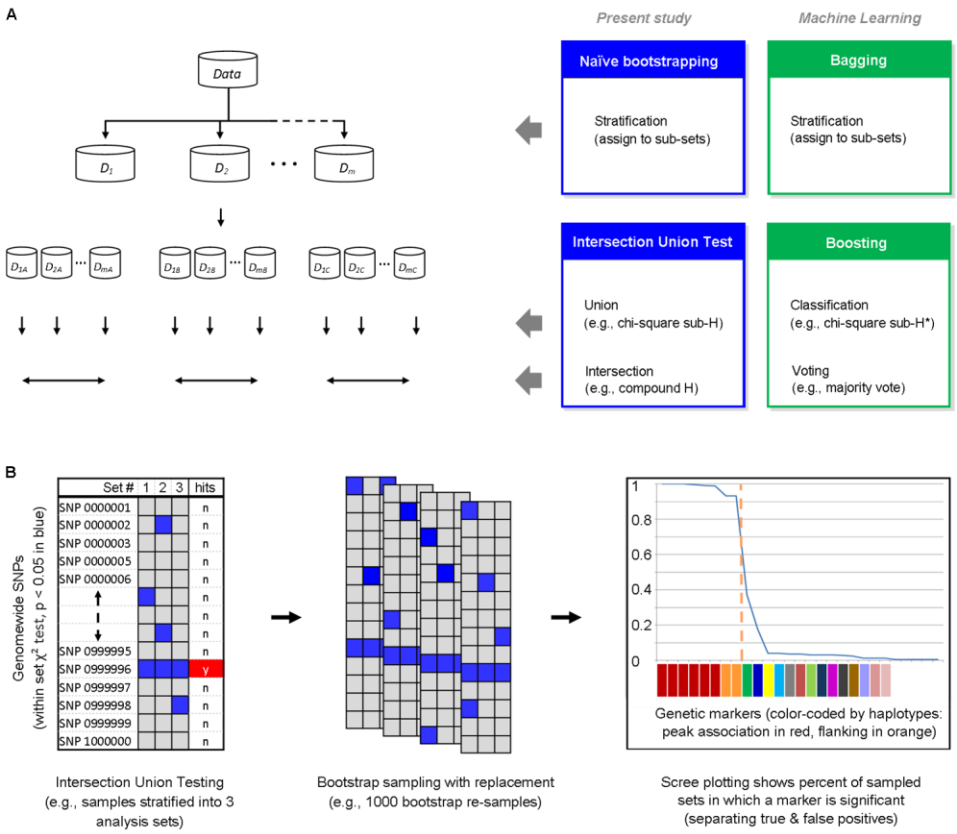

Figure 1. Schematic of integrated Bootstrapping and Intersection Union Testing (IUT) for genetic analysis. (***A***) The schematic on the left shows how a single dataset is repeatedly subsampled (with replacement) and each subsample of cases and controls is then put through the IUT compound hypothesis: i) for each subset of an IUT group, which genetic markers have statistically significant frequency differences in cases and controls, ii) keep only the markers that are significant in all subsets of an IUT (thus not requiring multiple testing correction). Right hand notations compare our methods, which are considered hypothesis tests, to analogous approaches in the field of Machine Learning, which are not considered hypothesis tests but rather learning or predicting. (***B***) Illustration of how IUT works in first panel: each marker (SNP) is tested for significance in each subset of an IUT group (set #1, 2, 3); only those significant in all are kept. Second panel illustrates how repeating the process on 1000 Bootstrap replicates (4 shown) can be used to plot the proportion of times a marker is positive in the 1000 (scree plot, third panel).

In this reporting year we discovered strong evidence that our method is very sensitive and specific based on analysis of the genetic contributions to the complex trait of dog size as a test (using the Vaysse et al. dataset cited above). Specifically, we reanalyzed that published data and, not only identified those authors' two genomewide significant hits using conventional methods, but we also found additional IUT-genomewide significant hits that they missed (but which have been shown to be true positives in other canine genetics studies). We also generated new evidence that Bootstrap/IUT methods i) have increased ability to detect weak signal (a critical need for complex genetics such as cancer risk) and ii) does not require correction for population structure when the analysis is designed properly. We did this by analyzing the most complex dog trait reported by Vaysse et al (ref. above) – sociability (the response of a dog when approached by another dog or a human) as a test (experimental support for these claims were provided in figures within the Q7 and Q8 Quarterly Reports).

I (Alvarez) have personally presented this methodology and our results to many investigators at international meetings. For example, at the premiere Gordon Conference of Human Genetics and Genomics (RI, 2013), I presented the methodology to several experts in diverse areas of genetics/genomics. That included arguably the most important population geneticist in the field of dog genetics, Dr. Carlos Bustamante (Stanford U.). He was intrigued by the method (specifically enquiring about its ability to handle population structure, which we address in our revised manuscript) and he was equally enthusiastic about the present project studying genetics in the military dogs. He expressed interest in discussing more and in collaborating. I also presented this work to several investigators at the 7th International Conference on Advances in Canine and Feline Genomics and Inherited Diseases (Broad Inst., Cambridge, MA, 2013).

Most importantly I presented this to one of the top two canine geneticists in the world, Dr. Kerstin Lindblad-Toh, Broad Inst. (Harvard U./MIT) and Karolinska Inst., and a leader of the LUPA consortium in Europe. She and her leading fellow in her group were both very interested in our methods and results, and we agreed to initiate collaborations. I also presented this work to Dr. Adam Boyko (Cornell U.), who is the fastest-rising investigator in the field of canine genetics. He was very interested, particularly in our success with the very complex trait of sociability in the Vaysse et al. dataset.

We are making the final revisions to resubmit the Rybaczyk et al. and Rowell et al. manuscripts. The former is only a matter of finalizing the writing and adjusting the figures (all analyses are complete). The latter may be complete, but the new collaboration with Dr. Lindblad-Toh (see above) has presented us with a second independent osteosarcoma positive and negative cohort of genotyped dogs of the same breed. We may therefore use that data to conduct further validation studies in order to make our findings more robust and higher impact.

We have already submitted a grant application to the National Institutes of Health on this topic (it was not funded) (Appendix I). Once our manuscripts above are submitted, we will revise that grant application and resubmit it.

Task 4 Identification, recruitment, and retention of cancer bearing and control dogs.

Among the findings from analyzing military dog records, we identified osteosarcoma, mammary cancer and mast cell cancer in the population. Examples of the importance of pedigrees follows: (Example i) One breeding program pair with litters within a very high-age group of 10-13 years of age (comparable to older than ~70 or more in humans) was shown to be potentially of high interest because she had cancer (as identified by being on the AFIP/JPC pathology report list noted above). However, the male appears to have been a "German police dog", a fact that would be important and has to be verified and considered in light of the experimental design (i.e., is that title a reference to origin an work application or is it a German Shepherd breed dog?). (Example ii) Another breeding pair with high-age litters is implicated as being important because it had two offspring in one litter that went on to develop cancer. These examples illustrate how we can triangulate on dogs of interest and controls by using pedigree information and AFIP/JPC pathology report data. In addition, we are examining the parents (and their other offspring) of those breeding pairs to evaluate the possibility of high and low cancer risk lineages.

In this section of the first annual report, we described how we validated the methods for genotyping of osteosarcoma and aged non-osteosarcoma dogs (using retired racing Greyhound DNA isolated from samples acquired through another funding mechanism and its associated IACUC approved protocol). There we also presented a figure (#1) of the analysis that used Principal Components Analysis to access separation of the population (which we classified by cancer status). We found significant difference in the genetic makeup of the two groups and were therefore satisfied with our selection protocol. That work is included in the Rowell et al. manuscript under revision.

In our second to last Quarterly Report (Q7), we noted that Task 2f involving dogs at a second military base (Fort Leonard Wood) was not necessary at that time. In the last Quarterly Report (Q8) that all the dogs and environmental aspects necessary are available from Lackland AFB. As we reported there, while the original grant application contained a letter of support from the veterinary service at Lackland AFB, they did not disclose to us significant information about their Military Working Dog program until the grant was funded. For that reason, we originally proposed to sample environmental differences by using dogs outside of Lackland AFB – and identified the population of Mine Program dogs at Fort Leonard Wood as an ideal control group exposed to a different environment than dogs at Lackland AFB. Having Ms. Perez, our Vet Tech, embedded in the veterinary service of the military dogs at Lackland AFB has allowed us to evaluate this environmental aspect. We have concluded that the Lackland AFB dogs have been exposed to various locations that range from uniform (housing at Lackland AFB Medina Annex) to common or unique deployments (from which some dogs are returned to Lackland AFB or Medina Annex temporarily or long term).

Task 5: Molecular Characterization of cancer bearing and control dogs

Highly detailed description and discussion of this was provided in the first year annual report. That described our development of the appropriate molecular protocols for acquisition, maintenance, and use of canine samples. In this reporting year, we developed the downstream portion of this analysis.

We developed two new aspects of sequences analysis in both tumor-bearing and non-tumor bearing dogs. i) We optimized high-throughput product purification (including DNA, RNA, and PCR product) for rapid sequencing. One time consuming step of the laboratory output is working with individual samples. We have thus far optimized a protocol that produces high-quality DNA, RNA, and PCR products at an individual sample level. However, as we begin to increase sample number and identify variants of interest (SNP array results generated through bioinformatics analysis), it will become necessary to validate this on large scale of dog samples. ii) We optimized the sequencing approach to validate SNP candidates and to genotype relatively small numbers of samples (<40). Larger numbers of samples will likely be genotyped with custom TaqMan assays, but we have determined that sequencing is the most flexible and cost-effective

approach for smaller numbers. By sequencing both strands, we can essentially guarantee 100% accuracy (otherwise we would refer to a nucleotide site as ambiguous or "suggestive/sequence evidence on one strand").

In a previous study, we (Alvarez) identified the genetic variant responsible for the brindle coat pattern in dogs. In the Rowell et al. study mentioned above, we therefore used brindle as a positive-control trait to test whether we could use the IUT/GIA to map that known locus in our Greyhound cohort (the same that was subsequently used to map osteosarcoma risk loci). That was a great success as the nearest polymorphic SNP in the cohort was the top brindle-associated SNP. We further examined the canine brindle locus as an opportunity to develop the molecular modalities planned for year 3. We optimized the Bisulfite sequencing protocol on genomic DNA and Real Time PCR on RNA. We narrowed down the methylation status of regions implicated for their cellular regulation roles to a single specific region of interest. We correlated DNA methylation status and mRNA expression levels. That work seems to show a very rarely observed mechanism whereby a long non-coding RNA in the antisense orientation regulates another gene epigenetically. As a result, we even generated a new protocol that allows for using qPCR to test strand-specific expression levels of RNA. That work is presently on hold, but will be followed-up for publication in the future.

Lastly, we have developed approaches to conduct cross-breed analysis. This is important if related breeds share a phenotype that is relatively rare in other breeds. If so, the most important variants associated with the phenotype are likely to be shared in the related breeds but not the other breeds (or at far reduced frequency). To that end, we conducted analysis of candidate osteosarcoma risk SNPs (from Greyhounds) in the closely related breed Scottish Deerhound (using samples acquired through other funding mechanisms and associated IACUC-approved protocol). Because SNPs are binary markers, single SNPs are not sufficiently informative to support haplotype sharing. We thus use canine SNP data to identify the nearby DNA sequence with the highest density of SNPs and use that to design ~500-600 bp PCR assays that include 2 or, ideally, more polymorphic SNPs.

Task 6- Adaptation of existing resources, data storage and hosting:

We have a secure virtual machine called Research DAPER or resdaper developed initially by Mr. Camerlengo and continued by his replacement Mr. Aaronson (supervised by Drs. Alvarez and Huang). The machine exists on the secure NCHRI (Alvarez) network behind a firewall. It can only be accessed by highly-secure VPN using two factor authentication. We have an instance Microsoft SQL Server stored on the machine. Microsoft SQL Server is an industry-leading relational database product that we use to store all of our documents after they have been digitalized. With a relational database, you can quickly compare information because of the arrangement of data in columns. The relational database model takes advantage of this uniformity to build completely new tables out of required information from existing tables. In other words, it uses the relationship of similar data to increase the speed and versatility of the database. The "relational" part of the name comes into play because of mathematical relations. Each table contains a column or columns that other tables can key on to gather information from that table. We have many fields that we can filter and sort on that we can use to retrieve items. Ultimately, this will include all clinical and associated data, environmental data and genetic (genotype), epigenetic, and genomic/molecular (phenotype) data. The user interface is under construction. We will have a web user interface that can be accessed by those with secure credentials. We have used Microsoft asp.net MVC to build the user interface. Using the model view controller pattern gives us the benefit of separating the representation of information from the user's interaction with it .The model consists of application data, business rules, logic, and functions. A view can be any output representation of data, such as a chart or a diagram. The controller mediates input, converting it to commands for the model or view.

In Task 2(i) we discussed the conversion of paper health records to digital versions using ABBYY software – mainly the 1829 form and the AFIP/JPC pathology reports. That digitized data will be fully accessible and searchable through the web interface mentioned above. In addition, the Task 2(ii) scanned complete veterinary clinical records will be directly linked as PDF format. This will allow analysis of digitized data with the option of follow-up detailed analysis of full health records on the same database/tools ensemble "resdaper" (or confirmation/cross-validation of critical data). We have thus installed the ABBYY FlexiCapture software and all of the components which include The Processing Server. That is the server that controls the operation of the Processing Stations. We installed the Licensing Server, the server that stores and manages licenses. We installed the Application Server, the server that controls the operation of the other components. We installed the Application Server components, which will allow operators to connect to the server and work using a web-browser. We also have the Application Server component which allows operators of web stations to register with the system and create requests for access rights to the web station. It provides operators of web stations with a single entry point into the system.

Task 7: Pathway analysis and functional characterization.

Task 7a is complete. I (Alvarez) have been conducting extensive data mining and analysis that are honing those skills which will ultimately be applied to the study of cancer in military dogs. That includes work on osteosarcoma risk candidate genes from Greyhounds (to be published in Rowell et al. manuscript mentioned above) and LUPA candidate genes for multiple canine traits (also discussed above). Most importantly, the Greyhound study implicated small genomic regions with one or two genes each. This allowed use of human cancer data and analysis servers to predict which were likely to be cancer genes and whether the human evidence suggested the cancer risk gene variant was likely to result in up or down regulation. For example, the IntoGen server permits analysis of gene expression and genome alterations associated with diverse cancer types. But other analysis servers, such as NextBio, Oncomine, KMplot and BioGPS provide different tools to mine the same gene expression data in very different ways. For example NextBio make meta-analysis of any subset of studies and KMplot generates Kaplan Meier survival plots for a subset of cancer types that have very large numbers of data available. With this data in hand, it is possible to generate hypotheses and to conduct cross-validation studies. For example, in the Greyhound osteosarcoma case, we can test those predictions by analyzing genetic association candidates in a canine osteosarcoma tumor gene expression dataset which includes Greyhound, Golden Retrievers, Rottweiler's and mixed breed dogs. Because there are orders of magnitude more human data than canine, it is critical to be able to make use of it.

Among the major aspects of genetic/genomic studies are contextualization according to biochemical or genetic pathways, cross-dimensional/platform validation, and comparative genomics/cross-species validation. To that end, I have conducted studies in these aspects of cancer genetics. Among those, I mined for genetic evidence that the enzyme aldehyde dehydrogenase is involved in multiple myeloma (for which there is experimental evidence generated by a collaborator studying this with their own funding). As a result of the latter analysis, my analyses were added to a manuscript that was recently accepted for publication. Although the following work was not based on our military dog data, my contributions involve the same analyses that will be conducted with canine cancer candidate genes: Yasmeen R., Meyers J. M., Alvarez C. E., Thomas J. L., Bonnegarde-Bernard A., Alder H., Papenfuss T. L., Benson D. M. Jr, Boyaka P. N., Ziouzenkova O. (2013) Aldehyde dehydrogenase-1a1 induces oncogene suppressor genes in B cell populations. Biochim Biophys Acta 1833:3218–3227. (See Appendix II) For example, I conducted the analysis shown in Figs. 6A and 6C. That critical information shows that the biology suggested by the Yasmeen et al. molecular/biochemical study can be cross-validated by public datasets involving other types of evidence (here gene expression). Similarly, we expect that the vast data available on human cancers will yield supporting evidence for canine cancer findings from the project that is the subject of this report.

Task 8- Project management, Quality control and assurance, and Security:

The most important change in this reporting year is the execution of the CRADA's which allowed us to acquire DoD military dog data. We established a footprint at Lackland and implemented security protocols in accordance with our agreements. We are conducting quality control evaluations for our data collection techniques to assure that we are collecting appropriate data. Once we have assured high quality data we will begin automated import into the database. We are also cross-validating medical and pathology records to assure accurate diagnosis. We initiated collaborations with Dr. David Gutman at Emory University and hope to use his automated pathology data base to facilitate confirmation of sample classification.

As of June 1st, 2013, Task 8 duties attributed to Dr. Rybaczyk (who has moved on in his academic career, as an NIH T32 Fellow, Michigan State U.) are being done by Dr. Alvarez. This transition was been smooth. A job listing was posted for a replacement postdoctoral fellow. Dr. Alvarez interviewed a highly-qualified postdoctoral fellow named Dr. Sohan Lal (currently postdoctoral fellow at Yale), but unfortunately Dr. Lal was forced to accept another position at Yale due to imminent expiration of his visa status. There is another candidate under consideration; the goal is to hire that person prior to initiating the biological sample collection.

The replacement for Mr. Camerlengo – computer programmer – was a success. His role has been taken up by Mr. Jacob Aaronson, who may not be as experienced as Mr. Camerlengo but appears to have greater affinity for the biomedical aspects of computational sciences. Mr. Aaronson quickly completed his NCHRI orientation, security clearance/ID badge, and vaccination requirements. Most importantly, he rapidly oriented himself in the project and is performing high quality work.

Key Research Accomplishments

- Execution of institutional agreements (CRADA's) between NCHRI (Alvarez)/OSU (Huang, Couto)

- Completion of all facets of IACUC between NCHRI and Lackland AFB through final Lackland AFB oversight approval (currently waiting for final ACURO approval expected within ~1 month)
- Successful embedding of NCHRI (Alvarez) Veterinary Technician, Ms. Michelle Perez within the military dog health service at Lackland AFB
- Successful scanning of veterinary clinical records by Ms. Perez at Lackland AFB, transmission of encrypted data to NCHRI, and uploading to DAPER database
- Continued development and validation of a scale free, high-power statistical methodology capable of resolving signal from noise in high throughput genetic/genomic data (IUT/GIA) by incorporation of Bootstrapping
- GIA manuscripts continue to be refined since receiving comments from peer reviewers
- GIA grant application to NIH is being refined based on peer reviewer critiques
- Expansion of our highly flexible data-infrastructure that is robust enough to handle military working dog records and queries of said records
- Initiation of high through-put software customization (ABBYY FlexiCapture) for analysis of 1829 longitudinal veterinary records and AFIP/JPC pathological records
- Initiation of DoD military dog pathology reports to identify cancer bearing dogs for cancer classification and selection of cases and controls
- Initiation of DoD military dog "puppy program" pedigree analysis for identification of high and low cancer risk lineages
- Development of a collaboration with Dr. Kerstin Lindblad-Toh, Broad Inst. (Harvard U./MIT)

Reportable Outcomes

- Dr. Jennie Rowell, having received her PhD from OSU for her work at NCHRI (Alvarez), joined the lab of one of two pre-eminent dog geneticists in the world, Elaine Ostrander, NIH, as postdoctoral fellow. The first week of Nov. 2013, she has a job interview for a tenure track position at the College of Nursing, OSU
- Expansion of DAPER database capabilities maintaining strong security
- Dr. Rybaczyk moved on to take an NIH T32 Fellow position, Michigan State U. (from which he is expected to be promoted to faculty once he acquires grant funding within approximately one year)
- Publication of Yasmeen et al. manuscript which included Dr. Alvarez's powerful cancer datamining and analyses that is similar to what will be applied to the military dog cancer studies in year 3
- Participation by Dr. Alvarez in the leading international Gordon Conference of Human Genetics and Genomics (which requires one to submit an application and be selected as an active contributor to the field) (RI, 2013)
- Participation by Dr. Alvarez in the 7th International Conference on Advances in Canine and Feline Genomics and Inherited Diseases (Broad Inst., Cambridge, MA, 2013)
- Dr. Couto retired from OSU effective Sept. 1st, 2013 and created his own consulting company
- Dr. Alvarez was promoted to Associate Professor with tenure by OSU
- Dr. Alvarez was offered a Scientist position (equivalent to Associate Professor) at the Cancer Center, Sanford Research, Sioux Falls, SD. [Discussions are ongoing, but he is happy at NCHRI/OSU and plans to stay through the completion of this project.]
- Dr. Alvarez's Center Chair and Division Head endorsed his application for leadership training [OSU College of Medicine's Center for Faculty Advancement, Mentoring and Engagement (FAME) 2014-2015 Faculty Leadership Institute] (application pending)

Conclusion

The project accelerated when the CRADA's were executed. In the first two years, we optimized the primary genotyping and molecular methods, and the follow-on validation methods. We also expanded the capabilities of our highly-flexible DAPER database and software tools in the present reporting year. In the first year we invented an entirely novel approach to conducting genome wide genetic association (GWA) analysis – genomewide IUT analysis (GIA); and in the second year we further validated it. In this second reporting year, we integrated IUT and Bootstrapping as an additional innovation with outstanding utility. Dr. Alvarez's presentation of these methods and results to leaders in the fields of genetics and canine genetics resulted in uniformly positive feedback from them (and multiple requests for collaboration). We expect to publish the two revised manuscripts on GIA (one on methods, one on empirical cancer mapping) shortly, but

the latter may be delayed while we analyze new supporting data acquired from Dr. Lindblad-Toh. In addition, we co-authored (Alvarez) a published study that was not based on the present military dog project, but which made use of the same datamining and analysis methods that will be used in our study. Dr. Rowell, one of our investigators (originally as a predoctoral student), moved on to conduct a postdoctoral fellowship with a pre-eminent dog geneticist at NIH and, after only a year there, is being recruited for a tenure track faculty position at OSU. Dr. Rybaczyk, another of our investigators (originally a postdoctoral fellow and promoted to research scientist) went on to be an NIH T32 Fellow at MSU, which is essentially a pre-faculty position. Dr. Alvarez was promoted to Associate Professor with tenure by OSU and is now under consideration for leadership training in the OSU College of Medicine.

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Appendices

I. Submitted National Institutes of Health grant application including Intersection Union Testing methodology (Aims 2, 3): Statistical techniques for optimized design and power in high-content genomics (Alvarez, PI; Huang, co-PI).

II. Accepted publication: Yasmeen R., Meyers J. M., Alvarez C. E., Thomas J. L., Bonnegarde-Bernard A., Alder H., Papenfuss T. L., Benson D. M. Jr, Boyaka P. N., Ziouzenkova O. (2013) Aldehyde dehydrogenase-1a1 induces oncogene suppressor genes in B cell populations. Biochim Biophys Acta 1833:3218–3227.

Descriptive Title: Statistical techniques for optimized design and power in high-content genomics

Submission Title:

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Opportunity Title: Exploratory Innovations in Biomedical Computational Science and Technology (R21)

Agency Name: National Institutes of Health

2. SPECIFIC AIMS. This application is in response to PAR-09-219, Exploratory Innovations in Biomedical Computational Science and Technology; it address research, development and application of analytical and statistical tools for interpretation of large biological data sets, and associated software. The flood of biological data has highlighted limitations to signal detection. Here we propose that combining optimized experimental design and novel uses of statistical methods can dramatically increase the power of signal detection. These approaches will be applicable to myriad data types and their integration. However, this proposal will demonstrate validity using a highly innovative approach to complex genetics. We will conduct a Genome Wide Association (GWA) study using high density genotyping that not only provides binary single nucleotide polymorphism (SNP) allele data, but also total SNP signal and allele ratios (which can be affected by DNA copy number variation, CNV). In Preliminary Studies we demonstrate the feasibility of using allele ratios as continuous variables to map disease loci. This is the first such GWA study of comprehensive CNV information without prior classification of markers as CNV. Our ***hypothesis*** is that implementation of our algorithm on multiple (experimentally standardized) groups dramatically increases the power to detect biological signal.

Experimental design. The now common use of thousands or tens of thousands of subjects in genetic studies can be attributed to genetic heterogeneity/complexity and diverse confounds of meta-analysis. A major limitation is the extreme multiple-testing burden in GWA, which is commonly done by Chi-Square testing of one million markers. In Preliminary Studies, we address these issues by 1) conducting complex disease mapping studies in one dog breed, which has 100-fold reduced genetic variation compared to humans, and 2) using multiple, but experimentally identical, case-control sets or batches. In this way, there are reduced numbers of disease-associated markers in a simpler background and we can apply an Intersection Union Test (IUT) across experiments (in place of Bonferroni multiple-test correction). ***Computational statistics.*** The overarching goal of the proposed analytical approaches is based on the information theory concept that the more manipulations or corrections are implemented, the more information is lost. We propose here that this loss of information can be eliminated in diverse types of biological data by integrating two elements. In the first, we use analysis of covariance (ANCOVA) to correct continuous variable data for latent known biological confounders such as group membership. In the second, we make use of optimized study design (specifically, using multiple case-control groups for a given experiment) to perform IUT. Others recently validated a similar use of IUT independently. In Preliminary Studies, we demonstrate validation of the integrated ANCOVA and IUT. We confirm that the use of IUT on multiple sets is a more effective solution to the three reversal paradoxes (Yule-Simpson, Lord's, and suppression) which share the characteristic that the association between two variables can be reversed, diminished, or enhanced when another variable is statistically controlled for. Notably, we are first to address these in the context of continuous genomic variables.

Aim 1: ***Demonstrate on large datasets the ability of ANCOVA to correctly identify biologically relevant phenomena that are linked to a disease trait.*** ANCOVA has been applied to correct for baseline variables in various fields, such as psychology and epidemiology. Despite similarities in variable types, data structure, and confounds, ANCOVA has never been applied to large scale genetic datasets. We will analyze different types of genomic datasets (our own and from the public domain) with well-established population confounds and show that ANCOVA is the most effective way of removing those.

Aim 2: ***Application of IUT for genetic analysis, allowing for multiple corrections without manipulation of individual datasets.*** We propose to demonstrate the ability of IUT to detect complex genetics in a disease phenotype and how combining IUT with ANCOVA will allow the detection of genetic determinants. The non-obvious advancement of this method is that it incorporates information theory by minimally altering the data before analyzing it. This retains the maximum amount of information for each measure. It also does not assume linear relationships with latent variables.

Aim 3: ***We will validate our claim that ANCOVA and IUT are more powerful than traditional techniques.*** We will replicate a published canine complex-genetics mapping study using fewer individuals to demonstrate that our technique is able to detect the same loci in addition other variants missed by traditional techniques. We will also conduct a novel GWA study of a human medically relevant complex trait in a second dog breed.

3. RESEARCH STRATEGY

(a) SIGNIFICANCE

We will develop and implement analytical and statistical tools (and software) for interpretation of large biological data sets. The explosion of biological data has made prominent several limitations to signal detection.[1] We demonstrate in Preliminary Studies that combining optimized experimental design and novel application of statistical approaches can dramatically improve signal detection. These methodologies will be applicable to analytical challenges of myriad data types and their integration [2], including genomics [3], high throughput (HT) sequencing [4], population biology and genetics [5,6], and gene/organism/environment interactions [7]. The improvements described here address the basic concept of information theory that more manipulations of data equals more information loss. Among the areas addressed, are 1) application of analysis of covariance (ANCOVA; [8]) to correct continuous variable data for latent known biological confounders as well as potentially avoiding the three reversal paradoxes (Yule-Simpson, Lord's, and suppression), which share the characteristic that the association between two variables can be reversed, diminished, or enhanced when another variable is statistically controlled for [9,10], and 2) multiple new applications of the Intersection Union Test (IUT; [11]), including GWA, as was independently developed by another investigator very recently [12]. This proposal thus offers solutions and software to address critical barriers to genomic analysis, simultaneously improving scientific knowledge and technical/analytical capabilities.

(b) INNOVATION

Multiple phenotypic traits (such as height or weight) are often treated as independent from the effect under study, but that neglects the reality that many traits are linked to other genetic and environmental modifiers. Others incorporate and calculate variances based on environmental or geographic stratifications. However, this ignores synergism between the organism, its immediate surroundings, and the greater environment. While it is not possible to measure and analyze every part of the environment, some baseline state must be identified from which deviation can be measured to test *a priori* hypotheses. In the absence of this uniform baseline, almost all statistical measures will fail to adequately detect regions of interest. ***This application will demonstrate feasibility and innovation in preliminary studies (c.5) using an entirely new approach (ANCOVA/IUT) to conducting genome wide association (GWA) genetics based on continuous variable data.*** An important challenge to GWA that relates to these issues above is population structure (i.e., correcting genetic studies for non-disease-associated allele frequencies that vary in human populations). Two common ways to address this are traditional meta-analytic techniques and IUT. But these approaches are selected more out of necessity than experimental design concerns. The majority of combinatorial studies have focused on publicly available datasets. Each of the individual datasets contains differing degrees of artifactual bias and other, potentially unrelated, variables. Oncomine's [13] and other algorithms applying this strategy to gene-expression have some success but it has not been the panacea originally prognosticated.[14]

Multivariate and integrative analyses can potentially solve many issues associated with genome wide studies.[15,16] However, they are limited by their ability to synthesize data into useful parcels of information that are applicable clinically or to research. Integrative analysis has the benefit of alternative testing. While multiple testing using the same measures and techniques increases error rates [17], alternative testing allows measurement of the same effect using different types of measures. As these are subjected to different analytic techniques, the posterior probability of false positives is reduced. Even with this strength, it is limited by biases and assumptions associated with individual measures. Ultimately the question of how to appropriately identify genetic contributions independent of latent confounds has not been conclusively answered. The gold standard for analyses is univariate testing. While geneticists talk about penetrance in relation to populations and percentages, the statistical actuality is that penetrance describes odds ratios. Establishing causation and deviation from population norms using case-control, linkage, or association analyses requires certain assumptions to be accepted that biologically may or may not be perilous to the analysis. While this is important to ethologists and population geneticists, attempting to compensate/account for these phenomena hinders and complicates analyses. We are interested in identifying biological outcomes that are well described and were

not concerned with tangential characteristics of the effect. To this end, we sought to isolate rather than compensate for effects. When examining multidimensional data it is easy to disregard the interaction of dimensions. Most dimensional reduction techniques measure and condense data so that interdimensional effects can be quantified. Priming effects can drastically alter these techniques and limit their usefulness. For this reason we applied ANCOVA [8] to remove independent effects from dependent effects prior to dimensional reduction. Here we show adjusted and un-adjusted measures to illustrate how the application of ANCOVA prior to traditional techniques is capable of increasing the sensitivity of a study, as well as the potential to correct for the reversal paradoxes (c.5. P.S., Study Design) by comparison to traditional normalization techniques.

(c) APPROACH

c.1. Research team. The multidisciplinary team is ideally suited for this project. Dr. Alvarez (PI) is PI in Molecular and Human Genetics, Nationwide Children's Hospital Research Institute, with a tenure track academic appointment at The Ohio State University College of Medicine. He has extensive expertise in molecular and human genetics and genomics, bioinformatics, and, from management level industry experience (Novartis Research), the discovery and validation of new drug targets and biomarkers. Dr. Leszek Rybaczyk (Research Scientist, Alvarez Lab) is expert in statistical bioinformatics. Dr. Huang Kun (Co-I) is co-director of the OSU-CCC Biomedical Informatics Shared Resource. His research is focused on developing bioinformatics tools for systems biology and research. Here he will be responsible for developing and implementing the software package. The advanced statistics expertise will come from a long term collaborator of the three investigators named above, Dr. Pramod K. Pathak (consultant, MSU). He is a theoretical and applied statistician with specific interests in statistical methods and their applications to biomedical research, sampling and resampling methods, computational statistics, reliability, and optimization problems in statistics.

c.2. Research strategy (RS). ***Note:*** As the approach has statistical components addressing different biology, we will explain the approach once, in Research Strategy, and establish feasibility in Preliminary Studies.

RS Aim 1. We propose to address these gaps by applying statistically proven methodologies in novel ways. ANCOVA has been applied in various fields such as psychology [18] and epidemiology [19] to correct for baseline variables.[20] Despite the similarities in variable types, data structure, and problems with confounds [19] ANCOVA has never been applied to large scale genetic datasets. **Aim 1:** Demonstrate on a large dataset the ability of ANCOVA to correctly identify biologically relevant phenomena that are linked to a disease trait. The rationale and technical approach for this aim are well elaborated in c.5. Preliminary Studies. Canine genetic data similar to those generated in Preliminary studies will be generated from 1) 36 Scottish Deerhounds: 18 osteosarcoma cases and 18 controls (i.e., three case-control batches of six and six), as well as 2) 36 Doberman (18 with cervical spondylomyelopathy and 18 controls (i.e., three case-control batches of six and six). In addition, we will analyze diverse genomic datasets from the public domain (including human SNP GWA, gene expression, and HT-sequencing). For example, by using TCGA data, in which the same patient's tissue was assayed on different microarrays in different laboratories, using an ANCOVA approach we will identify the most biologically relevant factors. We will expand that by looking not only at the cancer type, but also at the laboratory where the tissue was processed; the date on which it was processed, etc., and identify/potentially remove such intrinsic errors.[21] ***Power analysis.*** Based on our ongoing genetic studies (see Preliminary Studies), we assumed that potentially relevant SNPs will reduce the total of 173,000 SNPs to 1700 [MD Anderson Bioinformatics server with power of 0.8, acceptable false positives of 1, SD of 0.7. With the sample size of 36 dogs in each breed (18 cases and 18 controls) we will have 80 % to detect 2-fold differences in B allele frequency between cases and controls for candidate SNPs of interest (per SNP alpha = 0.00059). This is conservative, as ANCOVA and IUT would only reduce the variance.

RS Aim 1 Potential pitfalls and contingencies. (1) A limitation to using the integrated ANCOVA/IUT on biological data is that it is only applicable for continuous variable data. While this excludes, say, conventional binary-genotype GWA analysis, we address this need with the development of an IUT-alone approach; this use is now validated by us (see c.2. RS Aim 3 Expected results, Example 1) and by a second independent group.[12] Moreover, much genetic data (e.g., array CGH, HT-sequencing) and most genomic data has continuous variables (microarray and HT-sequencing based RNA expression and epigenetics, proteomics, metabolomic, etc.). (2) Another potential concern is the need for clear understanding of appropriate data structure. For that reason, we chose to make this proposal not only about the statistical methods, but also

about experimental design. We will make a major effort to document the proper use of these algorithms in publications and software Help documentation. (3) Lastly, these methods are computationally intensive. This will not affect us, as Dr. Huang (Co-I) is Director of Bioinformatics and has access to the OSU Supercomputer Center. Despite the computational demands, the methods proposed here offer analytical abilities that are unique and state of the art, and are sure to gain wide use. We believe that our optimization studies and careful statistical/software instructions will facilitate the most efficient implementation of our algorithms.

RS Aim 2. A second statistical technique, the Intersection Union Test, has been gaining use in the genomics field.[22] The IUT increases power, but also increases type I error as the number of comparisons increases.[12] However, because of the many latent confounds that cannot be accounted for in most genomic work, the IUT is the most elegant solution to reducing these errors.[23] For instance, in large datasets where a multitude of tests are conducted under traditional techniques, a multi-testing correction would need to be applied. However, as we previously demonstrated using the IUT, the probability of any specific false positive decreases exponentially with the addition of new datasets.[24] This is because the probability of detecting the same false positive in two independent datasets is the multiple of α, traditionally 0.05. For two datasets the probability of the same false positive being detected is 0.0025, for three it is 0.000125, and so on. This can compensate for even large datasets. In datasets with 173,000 variables (SNP arrays used in preliminary studies), using between 4 and 6 independent datasets would eliminate all false positives. Conversely if the same signal is being detected in 6 datasets the probability that it is due to chance is of the order $1.5x10^{-8}$. **Aim 2:** IUT is powerful new tool for genetic analysis and allows for multiple corrections without manipulation of individual datasets. We purpose to demonstrate the ability of IUT to detect complex genetics in a disease phenotype and how combing IUT with ANCOVA will allow the detection of genetic determinants and potentially explain penetrance. The non-obvious advancement of this method is that it incorporates information theory by minimally altering the data before analyzing it. This retains the maximum amount of information for each measure. The IUT is also not hampered by many of the assumptions of other tests.[20]

RS Aim 2 Potential pitfalls and contingencies. The IUT is dependent on having a common variable across all data sets used in the analysis. This variable can be very broad such as dog breed or very narrow such as a molecular phenotype. Regardless, the IUT will only answer questions related to the common variable among data sets. One way to correct for that is in the initial study design. The study design should take into account all of the limitations associated with the various statistical tests a priori. As we recently discussed in a publication, applying the IUT to unrelated data sets will result in the elimination of all signal.[24]

RS Aim 3 rationale. Large scale studies that use traditional GWA require large patient populations to achieve adequate power (and have yet to explain a significant portion of the heritability associated with most diseases).[25,26] This has serious pragmatic and ethical implications.[27] It also poses several experimental design problems as independent irrelevant variables – e.g., in genetics, population structure, can overpower the effect of interest.[28] Manipulation of data by Principal Component Analysis (PCA) after merging, or applying normalizations, hinge on the assumption that the interactions are linear. If the interactions are non-linear, applying these corrections can make analysis more difficult.[29] **Aim 3:** We propose to demonstrate that ANCOVA and IUT are more powerful than the traditional techniques by identifying a study and replicating that study using fewer patients and demonstrating that our technique is able to detect the same signal in addition other variants missed by the more traditional techniques.

RS Aim 3 Genetic studies experimental plan. As we did in Preliminary Studies (c.5., using the same Illumina 173,000 SNP array), we will conduct GWA analysis of two complex traits, each with high incidence in a dog breed. ***Mapping (1)*** As validation of a complex trait that has been mapped using a conventional genetic approach and published, we will map osteosarcoma in Scottish Deerhounds (one locus of dominant effect with evidence of linkage (Z_{max}=5.766)).[30] The original work used a 4-generation pedigree where 60 Deerhounds were genotyped and the genotypes of 70 others were inferred, for a total of 130 dogs. We will replicate that study using the methods developed in this proposal to conduct GWA (ANCOVA/IUT on B allele frequency data and IUT on allele/genotype data) on 18 Deerhound cases and 18 controls (i.e., three case-control batches of six and six). ***Mapping (2)*** In order to immediately draw high impact attention to our innovative approaches, we

propose to conduct GWA of a prominent breed-specific complex-genetic condition with high human relevance – "wobblers" or cervical spondylomyelopathy in Doberman Pinschers (reported to explain 2.5% of proportional mortality in the breed).[31,32] We have been collaborating for over a year with Ronaldo da Costa, our OSU colleague who is a leading authority in this.[32] We are currently conducting pedigree analysis on ~1000 Dobermans (showing strong evidence of heritability; data not shown), and have initiated collection of blood/DNA samples. Using the Doberman wobblers pedigree, we will select optimal informative dogs to conduct a mapping study with 18 cases and 18 controls (i.e., three case-control batches of six and six). ***Power analysis.*** See c.2. RS Aim 1, end of first paragraph.***Follow up to broad mapping:*** depending on the type/strength of the evidence and the length of the haplotypes, we will conduct either fine mapping in related breeds that share a similar phenotype, sequence implicated haplotypes using sequence capture, or characterize transposition events, structural variation or DNA methylation status (see PI (Alvarez) biosketch, which demonstrates successful funding of grants in this area from NIH, DoD CDMRP and AKC-CHF). The PI is expert in genomics and sequence and evolutionary biology analyses that will be required to fully evaluate genetic variants and their possible disease effects.[32-38]

RS Aim 3 Expected results. We predict that in *Mapping (1)* we will identify the same locus published previously (leading to refining the locus through recombination in both breeds), and that we will identify other loci associated with osteosarcoma risk – both SNP alleles and B allele frequency changes suggestive of CNV or of effects resulting in allele-specific SNP genotyping bias from amplification step [[39]]. As Deerhounds are relatively closely related to Greyhounds, we also expect to find some loci shared between the two, which would provide convincing replication of the findings in our preliminary studies. We predict that in *Mapping (2)* we will find wobblers-associated variants. For both mapping studies we expect to identify loci that could not have been found using conventional genetic analyses. *Example 1*, in preliminary GWA studies applying IUT to binary genotype calling of the same Illumina SNP array data used in c.5. Preliminary Studies, we identified a genome wide significant locus that would not have been identified by conventional Chi-Square GWA analysis (not shown). Strikingly, two of the three case-control groups had increased frequency of the SNP allele associated with high risk, but the third group had reduced frequency of the same allele associated with reduced risk. We propose that, due to reversal paradox effects [[9,10]], many such findings cannot be detected by conventional GWA. We also expect to identify candidate genes (e.g., some osteosarcoma candidate haplotypes have no more than one gene) and variants (e.g., through sequence capture) within association loci. *Example 2,* in Preliminary Studies we demonstrate the use of ANCOVA/IUT to identify continuous variable differences in B allele frequencies associated with osteosarcoma risk. This would not be possible with current approaches that map binary SNP alleles (and cannot be detected indirectly by tag-SNPs in LD when the variants are relatively recent). Such variation may be indicative of genetic effects never before sampled genome wide for GWA, such as CNV or isothermal amplification bias [[39]] in Illumina Infinium SNP genotyping (e.g., due to DNA methylation, structural variation, and retrotransposition events). If our expected results materialize, as is strongly supported by our preliminary studies, they would establish the superior power and preservation of information in the innovative experimental design and analyses we propose; and it would open the door to studying the most common (and with highest mutation rates) types of genetic variation [[38]] for the first time.

RS Aim 3 Potential pitfalls and contingencies. Our preliminary studies support the feasibility of applying very well-established statistical methods for novel biological data analyses. For example, applying an IUT approach to GWA using binary genotype data, identified a SNP locus at genome wide significance; but no locus reached significance using conventional Chi-Square analysis on the same genotype data (see Example 1 in previous section). Notably, others have recently independently validated that same application of IUT.[12] A second example is the fact that the ANCOVA/IUT mapping approach identified several loci that were covered by multiple significant SNPs, including five SNPs in a 600,000 kb region of chr6; the odds of the observed physical genome distribution being a random effect are infinitesimally low. The greatest challenges in the field of GWA are validation of association and identification of causative mutations. These remain potential pitfalls for us, but we are encouraged by the fact that our osteosarcoma GWA (using IUT of conventional binary genotypes) in Greyhounds identified one (of 19 significant) SNPs within the 4.5 Mb interval identified for

linkage to osteosarcoma in the closely related Scottish Deerhound. This ability to fine map across related breeds is one of the major strengths of dogs, as are the reduced phenotypic and genetic heterogeneity.[40] For the mutation detection, we will be challenged as is everyone, but 1) we have improved chances over most others because we will have more loci to prioritize for specific molecular approaches based on our types of findings (say, structural variation vs. DNA methylation), and 2) we have the technical and computational expertise, and are using the most cutting edge methodologies.

c.4. Software development

All the algorithms developed in this project will be integrated into an open source R package using R and Bioconductor functions and packages. The package will be tested on both stand-alone workstation and also parallel computing environment including two clusters available at OSU (one in the Ohio Supercomputer Center, one in the Dept. of Biomedical Informatics). The packages will be released on a project website and freely available to public. In addition, we will submit it to Bioconductor in compliance with the testing and inclusion criteria. If time permits, we will also consider integrating the R package into a web tool using web interface tools such as the *Rcgi* package (a CGI WWW interface R).

c.5. Preliminary studies & Demonstration of proposed experimental approach

Note: To demonstrate the novelty and significance, and the experimental plan for all three Aims, we devote significant space in this proposal to describe our preliminary studies (two manuscripts in preparation)..

Study design (ANCOVA/IUT approach), canine osteosarcoma (OSA). Dog breeds have ~100-fold less genetic variation than humans. Greyhounds were split over one hundred years ago into racing and show sub-breeds (registered NGA and AKC, respectively). Strikingly, racers have the highest OSA rate (25% incidence) of any breed, whereas show dogs have no increased risk.[41,42] We thus designed a study of a complex genetic trait in an outbred mammal, but used one of the simplest such contexts possible. Genotyping of these dogs was performed using the highest density SNP array available in dogs (Illumina HD, 173,000 feature; fewer SNPs than humans due to the highly extended linkage disequilibrium (LD) in dogs). Importantly, this genotyping platform provides not only the presence or absence of the binary A or B alleles at each marker, but also the signal intensity of the marker and the ratio of the two alleles (referred to as B allele frequency, BAF). We conducted the SNP genotyping in three OSA positive-negative (case-control) groups in order to 1) using ANCOVA to adjust for group membership as well as potentially addressing the three reversal paradoxes (Yule-Simpson, Lord's, and suppression), which share the characteristic that the association between two variables can be reversed, diminished, or enhanced when another variable is statistically controlled for [[9,10]]; and 2) enable the use of IUT in place of GWA by Chi-Square analysis with Bonferroni multiple testing correction. Specifically, we genotyped batches of 12 dogs in the combination of 4 OSA racers, 4 OSA free racers (OFR) and 4 show (AKC).**Statistics & Results:** Data was analyzed using Illumina GS and Partek GS. Sample attributes (incl.

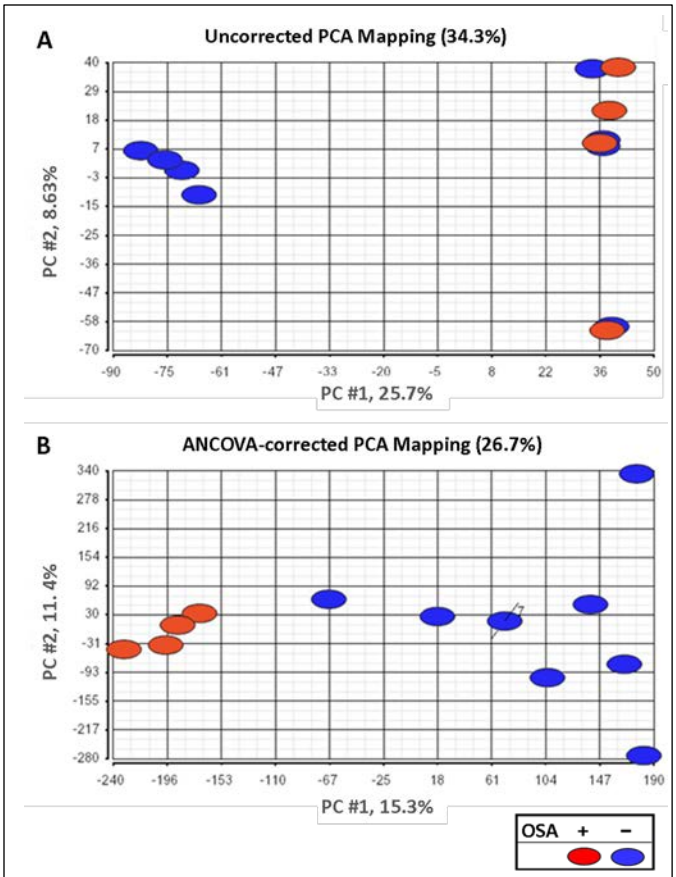

Fig 1. Application of ANCOVA. Correction of Greyhound osteosarcoma (OSA) positive and negative continuous variable genotypes (B allele frequencies). (A) Uncorrected analysis shows population structure effects: separating OSA positive and negative racers apart from negative AKC show Greyhounds. (B) ANCOVA-corrected analysis cleanly separates OSA positive and negative dogs.

racing/show and disease status) were used to assign animals to conditions for ANCOVA corrections. ANCOVA is based on regressions and when used as a statistical test assumes that covariates are independent variables. In our ANCOVA procedure we used it to establish weighted averages so that groups that are biologically similar have the same regression slope. Linear models in biological contexts have been heavily criticized. In this procedure a linear model is entirely appropriate since we are classifying based on known biological traits. Although this does render the measures arbitrary it allows for effects to be isolated that can be subjected post hoc to other tests. Figure 1 demonstrates the effects of ANCOVA isolation on principal components associated with the phenotype of interest. Before correction, two low risk groups (AKC and OFR) fail to cluster according to risk due to population structure. Regression lines were computed for the appropriate factors and interaction values were transformed and weighted to correct for the slope of the generalized linear model. We next calculated the covariance matrix of the loading values for each dataset and conducted IUT using a threshold of ±0.6. Many publications have reported that Pearson correlation (r) values of 0.4 are biologically significant. Here we used 0.6 assuming it most likely captures the most informative SNPs.

Table 1. Analysis of informative SNPs using ANOVA for multiple categories of risk.

SNP	Chr	Position
BICF2S23318678	3	22278940
BICF2P756511	3	34630563
BICF2S22958963	3	34806577
BICF2S23713946	5	3741194
G320f26S259	5	3814438
BICF2P959468	5	24064707
BICF2S23647041	5	25563084
BICF2S23746914	6	71831263
BICF2S22933176	6	72089371
BICF2P643804	6	72282176
BICF2P878053	6	72314083
BICF2S23332924	6	72453644
G439f54S214	7	23851944
TIGRP2P97627	7	49152204
BICF2P989771	9	27058611
BICF2P395540	12	67862864
BICF2P998637	14	39888317
BICF2S23147465	14	51418412
BICF2S2339350	18	23106621
BICF2S23348607	18	23130080
BICF2P950849	18	37553821
TIGRP2P335678	25	54551661
BICF2P691768	28	42235397
BICF2P681391	31	39698895
BICF2P623089	34	30054450

A list of potential candidate SNPs from the ANCOVA/IUT was identified and used to filter genotype information. Genotypes were subjected to a Chi-Square test of association for osteosarcoma risk. Non-significant genotypes were eliminated from the analysis. Once only SNPs that are loaded with the most meaningful measures remained we conducted t-tests to determine if they were capable of discriminating between the two training populations. This procedure revealed that the osteosarcoma free racers and the AKC show greyhounds which have below average incidence rate clustered together and the first principle component explained the osteosarcoma risk variability initially masked by the effects of the population difference (Fig. 1B). We then went on to determine whether it was a genotypic effect such as haplotypes or if some other mechanism was associated with the differential risk in these two populations. Intriguingly, regions associated with altered risk could not be identified based on haplotypes alone. However, the signal was derived from alterations in B allele frequency that correctly categorizing dogs across unrelated datasets. The genome wide significant hits are shown in Table 1. Encouragingly, several regions are detected by multiple SNPs (colored), including five SNPs in a 600,000 kb region of chromosome 6.

Preliminary studies conclusions. Here we presented the first GWA study of osteosarcoma in any organism, and reported approximately twenty hits. Our approach showed how population structure can affect the ability to detect biologically relevant genetic effects. In addition, this is the first work to detect genome wide significant association signal using continuous variable genotype data (B allele ratios) and ANCOVA/IUT; we propose those loci are a combination of CNVs and genetic/epigenetic variants with differing amplification bias [[39]] in the SNP genotyping protocol. This is consistent with Dr. Nadeau's suggestion that the missing heritability may lie in unexplored genome regions or "in largely untested classes of genetic variation."[43] Beyond the analysis shown here, we conducted a second GWA analysis of the same data, but applying only IUT using binary allele calls – see c.2., RS Aim 3, Expected results and Potential pitfalls and contingencies. That analysis suggested validation of the study, as one of 19 genome wide significant hits is within the 4.5 Mb interval linked to osteosarcoma in Deerhounds. Moreover, we identified SNPs that could not be identified by conventional approaches due to the reversal paradoxes.

Application summary: We propose to develop novel applications of validated statistical approaches to enable greatly improved analysis of continuous-variable biological data. This and the new applications of IUT will be widely used for genomic and integrative analyses.

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Aldehyde dehydrogenase-1a1 induces oncogene suppressor genes in B cell populations

R. Yasmeen [a], J.M. Meyers [a], C.E. Alvarez [b,c], J.L. Thomas [a], A. Bonnegarde-Bernard [d], H. Alder [e], T.L. Papenfuss [d], D.M. Benson Jr. [f], P.N. Boyaka [d], O. Ziouzenkova [a,*]

[a] Department of Human Sciences, The Ohio State University, Columbus, OH 43210, USA
[b] Center for Molecular and Human Genetics, The Research Institute at Nationwide Children's Hospital, Columbus, OH 43205, USA
[c] Department of Pediatrics, The Ohio State University College of Medicine, Columbus, OH 43210, USA
[d] Department of Veterinary Biosciences, The Ohio State University, Columbus, OH 43210, USA
[e] Nucleic Acid Shared Resource, Comprehensive Cancer Center, The Ohio State University, Columbus, OH 43210, USA
[f] Division of Hematology, Comprehensive Cancer Center, The Ohio State University, Columbus, OH 43210, USA

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ABSTRACT

The deregulation of B cell differentiation has been shown to contribute to autoimmune disorders, hematological cancers, and aging. We provide evidence that the retinoic acid-producing enzyme aldehyde dehydrogenase 1a1 (*Aldh1a1*) is an oncogene suppressor in specific splenic IgG1$^+$/CD19$^-$ and IgG1$^+$/CD19$^+$ B cell populations. *Aldh1a1* regulated transcription factors during B cell differentiation in a sequential manner: 1) retinoic acid receptor alpha (*Rara*) in IgG1$^+$/CD19$^-$ and 2) zinc finger protein *Zfp423* and peroxisome proliferator-activated receptor gamma (*Pparg*) in IgG1$^+$/CD19$^+$ splenocytes. In *Aldh1a1*$^{-/-}$ mice, splenic IgG1$^+$/CD19$^-$ and IgG1$^+$/CD19$^+$ B cells acquired expression of proto-oncogenic genes *c-Fos*, *c-Jun*, and *Hoxa10* that resulted in splenomegaly. Human multiple myeloma B cell lines also lack *Aldh1a1* expression; however, ectopic *Aldh1a1* expression rescued *Rara* and *Znf423* expressions in these cells. Our data highlight a mechanism by which an enzyme involved in vitamin A metabolism can improve B cell resistance to oncogenesis.

1. Introduction

The deregulation of B cell differentiation has been shown to play a causal role in autoimmune disorders, carcinogenesis, and aging [1]. B cells differentiate from B-lymphoid progenitors in the bone marrow and progress through many stages during differentiation to ultimately express B cell receptor (BCR). Lymphocytes expressing surface IgM migrate to the spleen [2], where self-reactive splenic B cells undergo apoptosis; others become responsive to T-cell-dependent and T-cell-independent antigens. The various B-cell populations are compartmentalized in different splenic zones, including red pulp, marginal zone, and white pulp. After pathogen exposure, they complete differentiation in germinal centers [2]. Specific populations of B cells can undergo alternative differentiation. For instance, purified mouse splenic B cells respond to stimulation with cytokines, anti-CD38, anti-CD40, anti-μ, and retinoic acid (RA) by enriching IgG1$^+$ and CD138$^+$ B cell populations and genes involved in the regulation of Ig somatic hypermutation and class switching [3]. In these B cells, RA contributed to the suppression of activation-induced deaminase (Aid), transcriptional regulators of differentiation (Pax5), and neoplastic transformation t(9;14) [3,4]. Oncogenic processes further diversify in B lymphoma cells [5]. The physiological mechanisms responsible for the formation of specific B cell subsets have remained unexplored.

The studies with dietary vitamin A (retinol or retinyl esters) highlighted a possible role for this pathway in specific B cell responses. Dietary vitamin A content influenced IgA production against T-cell dependent and T-cell independent type 2 antigens at mucosal locations [6]. Vitamin A deficiency in the diet diminishes immune responses and increases mortality [7–10]. These responses may be partially improved by supplementation with either vitamin A or its metabolite RA, arguing for RA as a mediator of these responses. The function of RA in B cell studies in vitro revealed that multiple aspects of B cell biology are RA-sensitive [10,11]. RA accelerated differentiation of a subset of proliferating lymphoid progenitor cells into B cells by targeting the oncogenes c-myc and cyclin D3, cytokines, and NFκB, as well as kinase p38/CDK2 [12,13]. RA treatment also promoted differentiation of malignant B cells, alone or in combination with rosiglitazone, an agonist for

Abbreviations: Aldh1a1, aldehyde dehydrogenase 1a1; AP1, activator protein 1 a heterodimeric transcription factor formed by *c-jun* and *c-fos*; c-Fos, transcription factor encoded by the FOS gene; c-Jun, protein encoded by *c-Jun* gene; *Hoxa10*, transcription factor homeobox protein a10; Ig, immunoglobulin; *Pparg*, peroxisome proliferator-activated receptor; RA, retinoic acid; *Rara*, retinoic acid receptor alpha; RARE, retinoic acid receptor response element; *Zfp423*, murine zinc finger protein; *Znf423*, human zinc finger protein

* Corresponding author at: 1787 Neil Avenue, 331A Campbell Hall, Columbus, OH 43210, USA. Tel.: +1 614 292 5034; fax: +1 614 292 8880.
E-mail address: ziouzenkova.1@osu.edu (O. Ziouzenkova).

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the nuclear receptor PPARγ [14]. The understanding of RA in immune function is incomplete. For example, a recent trial in Guinea–Bissau revealed a paradoxically higher mortality in girls supplemented with vitamin A than in placebo group [15]. In this study we dissected the role of endogenous vitamin A metabolism on gene regulation in B cells.

An increase in the intracellular RA concentrations is generated in response to various hormonal, dietary, and inflammatory stimuli and may be considered as a factor in endogenous differentiation of B cell subsets [11,16]. RA is produced sequentially. Alcohol dehydrogenases (ADH and SDR/RDH) oxidize retinol to retinaldehyde, which is dehydrogenated into RA by the members of aldehyde dehydrogenase-1 family of enzymes: ALDH1a1, ALDH1a2, and ALDH1a3 [17]. The principal mechanism of RA action is through the activation of RA receptors (RAR). RA binding to RAR induces its heterodimerization with retinoid X receptor, and binding to cognate response element (RARE) sequences in the promoters of target genes [18]. In addition, RA regulates a plethora of signaling and transcriptional pathways [19], including a *Zfp423*-dependent induction of *Pparg* expression [20]. Paracrine RA production by ALDH1 enzymes in dendritic cells plays an important role in B cell homing to the mucosa, and promotes IgA isotype class switching [21,22]. The role of RA-generating enzymes in B cells has remained unexplored. Here, we investigated the transcriptional function of ALDH1a1 in murine B cell subsets and in human multiple myeloma B cell lines.

2. Materials and methods

2.1. Reagents

We purchased reagents from Sigma-Aldrich (St. Louis, MO) and cell culture media from Invitrogen (Carlsbad, CA) unless otherwise indicated. Anti-mouse antibodies were: CD19 from BD Biosciences (San Jose, CA) and β-galactosidase from Abcam (Cambridge, MA).

2.2. Animal studies

All experimental protocols were approved by the Institutional Animal Care and User Committee. Water and regular chow (Harlan Laboratories, Indianapolis, IL) were available ad libitum in all mouse studies.

Study 1 employed Tg RARE-Hspa1b/lacZ (denoted as RARE-lacZ) reporter mice developed by Dr. J. Rossant using a transgenic construct containing 3 copies of the 32 bp RARE placed upstream of the mouse heat shock protein 1B promoter and β-galactosidase gene (lacZ) [23]. Female mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Three RARE-lacZ and three wild-type C57BL/6J (WT) female mice (12–15 weeks old) were fed regular chow throughout this study.

Study 2: *Aldh1a1*$^{-/-}$ mice were previously generated in the laboratory of G. Duester [24] and characterized for their metabolic responses [20,25,26]. *Aldh1a1*$^{-/-}$ (n = 10) and WT (n = 9) 13–14 month old male and female mice were used for these studies. Mice were fed regular chow diet. Blood was collected by cardiac puncture in EDTA-containing tubes. The spleens isolated from 3 randomly-selected females were used for IgG1^{+}/CD19^{-} and IgG1^{+}/CD19^{+} B-cell separation. The remaining spleens and other organs were used for other analyses.

2.3. Human cells

Leukopacks (American Red Cross, Columbus, OH) were obtained from healthy donors under an Institutional Review Board-approved procurement protocol. Peripheral blood mononuclear cells (PBMC) were cultured in RPMI 1640 media (Invitrogen) supplemented with 10% fetal bovine serum (ICN Biomedicals, Irvine, CA) and kept at 37 °C in a 5% CO_2/air incubator.

2.4. Flow cytometry analysis (FACS)

Splenocyte suspension was obtained from whole spleens dissected from 4 WT and 4 *Aldh1a1*$^{-/-}$ mice (2 males and 2 females in each group). Briefly, spleens were collected and mononuclear cell suspensions were prepared by mechanical disruption with the aid of a cell strainer (BD Biosciences, San Jose, CA) followed by brief incubation in NH_4Cl (0.08%) to remove red blood cells. Splenocytes were resuspended in fluorescence-activated cell sorting buffer (FACS; PBS containing 0.1% BSA and 0.1% sodium azide) at 5×10^5/100 μL. Cells were stained with a panel of antibodies from AbD Serotec (Bio-Rad Laboratories, Inc., Hercules, CA) and available isotype controls. All antibodies were primary, non-conjugated with the exception of MHC class II which were directly conjugated to fluorescein isothiocyanate (FITC) and Alexa Fluor 647 (BD Biosciences), respectively. Secondary antibodies of either phycoerythrin (PE; 5 μL) or fluorescein isothiocyanate (FITC; 1 μL) were purchased from AbD Serotec and were used at various dilutions (1:10, 1:50, and 1:100) [27]. Samples were analyzed using BD Accuri flow cytometer and analyzed with BD Accuri Flow analysis software (BD Biosciences).

2.5. Purification of IgG1/CD19^{-} and IgG1/CD19^{+} B cells

Splenic B cell subsets were obtained from four WT and *Aldh1a1*$^{-/-}$ female and one male mice. They were purified by automated magnetic cell separation (autoMACS, Miltenyi Biotec). The cell suspensions were incubated with microbeads conjugated with anti-mouse CD19. The CD19^{-} and CD19^{+} populations were separated by autoMACS. The CD19^{-} and CD19^{+} fraction was further incubated with a biotinylated rat anti-mouse γ1 (clone G1-7.3, BD Biosciences) and streptavidin-conjugated microbeads. Populations of IgG1^{+}/CD19^{-} and IgG1^{+}/CD19^{+} were separated by autoMACS. Throughout, we denoted these populations as CD19^{-} and CD19^{+}. The purity (>98%) of the cell population was confirmed by FACS.

2.6. RNA isolation and quantitative real time PCR (qRT-PCR)

Total RNA was prepared using the RNeasy kit (Qiagen, Valencia, CA). qRT-PCR was performed with predesigned assays (Applied Biosystems, Foster City, CA) using a 7900HT Fast Real-Time PCR System, TaqMan detection system, and validated primers (Applied Biosystems, Foster City, CA) in triplicate as described [16]. The mRNA expression was calculated based on Tata-box binding protein (TBP) expression for normalization using the comparative Ct method.

2.7. NanoString gene expression profiling

The digital multiplexed NanoString nCounter mouse inflammation expression assay (NanoString Technologies) was performed with 100 ng of total RNA according to the manufacturer's instructions. RNA was isolated from CD19^{-} and CD19^{+} fraction isolated from 3 female WT and *Aldh1a1*$^{-/-}$ mice. NanoString's nCounter technology is based on direct detection of target molecules using color-coded molecular barcodes, providing a digital quantification of the number of target molecules [28]. Total mRNA (5 μL) was hybridized overnight with nCounter Reporter (20 μL) probes in hybridization buffer and nCounter Capture probes (5 μL). The hybridizations were incubated at 65 °C for 16–20 h in excess of probes to ensure that each target finds a probe pair. Excess probes were removed using two-step magnetic bead based purification on the nCounter Prep Station. The hybridization mixture containing target/probe complexes was allowed to bind to magnetic beads containing complementary sequences on the Capture Probe and washed followed by a sequential binding to sequences on the Reporter Probe. Biotinylated capture probe-bound samples were immobilized and recovered on a streptavidin-coated cartridge. The abundance of specific target molecules was then quantified using the nCounter Digital Analyzer to count

the individual fluorescent barcodes and assess target molecules present in each sample with a CCD camera. For each assay, a high-density scan (600 fields of view) was performed at the highest standard data resolution, 600 fields of view (FOV) that is the dynamic range and level of sensitivity in the system. Images were processed internally into a digital format and were normalized using the NanoString nSolver software analysis tool. Counts were normalized for all target RNAs in all samples based on the positive control RNA to account for differences in hybridization efficiency and post-hybridization processing, including purification and immobilization of complexes. Subsequently, a normalization of mRNA content was performed using six internal reference housekeeping genes that were included within the mouse inflammatory panel: *Cltc*, *Gapdh*, *Gusb*, *Hprt1*, *Pgk1*, and *Tubb*. The average was normalized by background counts for each sample obtained from the average of the eight negative control counts. Counts were corrected by subtracting the mean and 2 times standard deviation value of the negative control from the counts obtained for each target RNA.

2.8. Immunohistochemistry

Spleens and kidney were embedded in paraffin. Immunohistochemical analysis of spleens from WT and RARE-lacZ mice was performed with rabbit polyclonal β-galactosidase antibody (1:1000 dilution). Images were obtained using Olympus M081 IX50 and Pixera Viewfinder 3.0 software.

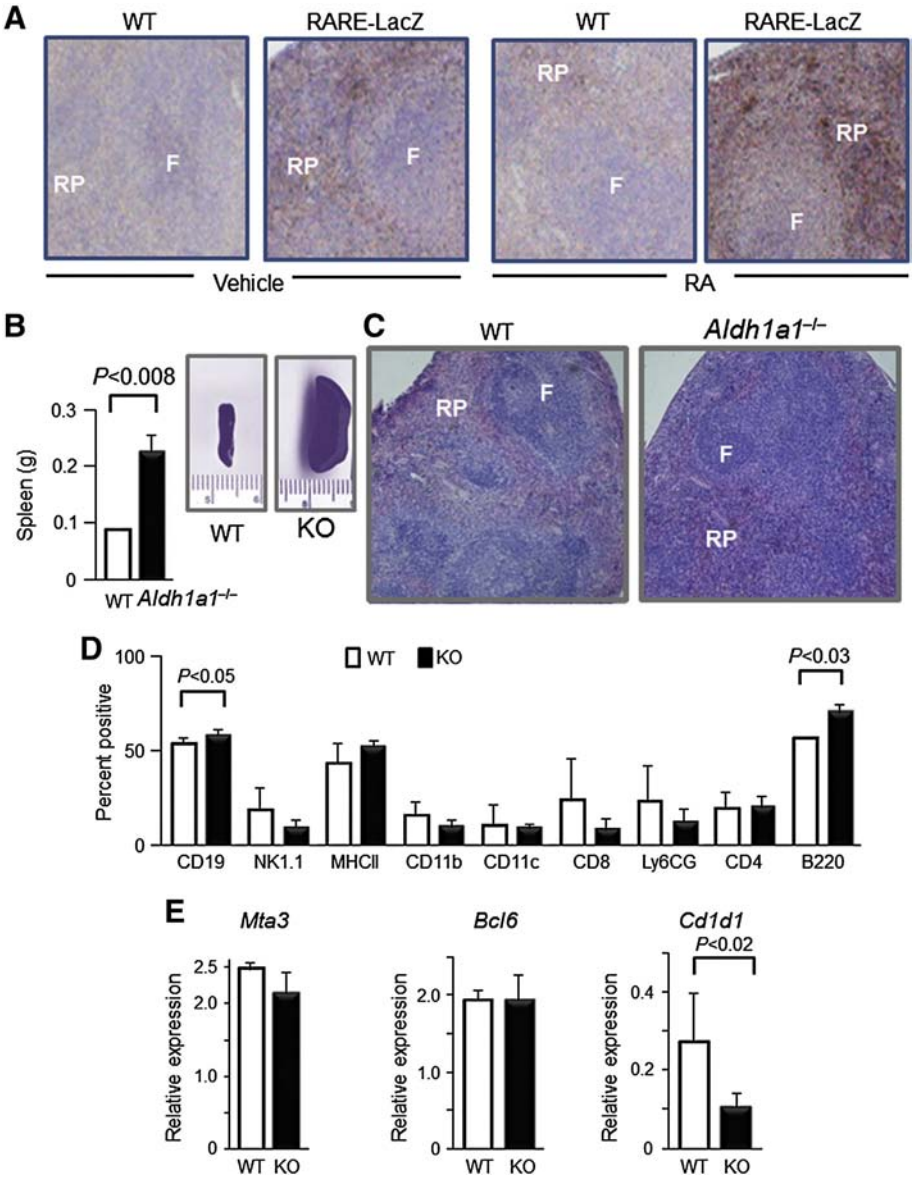


Fig. 1. Activation of retinoic acid receptor response element (RARE) accompanies splenic red pulp development, which depends on *Aldh1a1*. (A) RARE activation was studied in WT and RARE-LacZ mice (n = 3 from each group) which were injected every 48 h, up to a total of 3 injections with 1 mL PBS (vehicle) without or with RA (500 nM). RA was added into PBS from 500 μM RA stock solution in ethanol immediately before injection. Vehicle PBS solution contained 1 μL ethanol. All RA solutions were protected from light and stored under argon atmosphere. Total injected RA amount was 1.5 nmol per mouse (0.15 μg/dose). Immediately after the third injection, mice were harvested and their spleens were embedded in paraffin. Immunohistochemistry was performed with anti-β-galactosidase antibody. Heterogeneous brown β-galactosidase-positive areas were found in red pulp (RP) compared to follicular zone (F) (10× magnification). The RARE responses in adipose and hepatic tissues were described in [25]. (B) Weight of spleens (left panel, Study 2. *Aldh1a1*$^{-/-}$ (n = 10) and WT (n = 9)). Inset shows the representative whole spleen images of WT and *Aldh1a1*$^{-/-}$ mice. (C) Representative hematoxylin & eosin staining of paraffin embedded spleen section from WT and *Aldh1a1*$^{-/-}$ (KO) mice from the same study (n = 3 per group). (D) FACS analysis of splenocyte suspension isolated from whole spleens of *Aldh1a1*$^{-/-}$ (n = 4) and WT (n = 4) mice. *P*, significance levels, Mann–Whitney *U* test. (E) Expression of germinal center markers in the total spleen lysates isolated from WT (white bars) and *Aldh1a1*$^{-/-}$ (black bars) mice was analyzed by TaqMan assays (WT: n = 3; *Aldh1a1*$^{-/-}$ n = 5). Data were normalized by *TBP*. Significant difference was determined using Mann–Whitney *U* test.

2.9. Transfections

U266B1 (U266) and RPMI8226 were purchased from American Type Culture Collection (Manassas, VA), other B cell lines were provided by Dr. D.M. Benson, Jr. All human B cells were maintained in 15% fetal bovine serum/RPMI 1640 medium as previously described [29]. Human full length *Aldh1a1* cDNA expression vector was purchased from OriGene (Rockville, MD). U266 cells (6×10^6 per tube) were

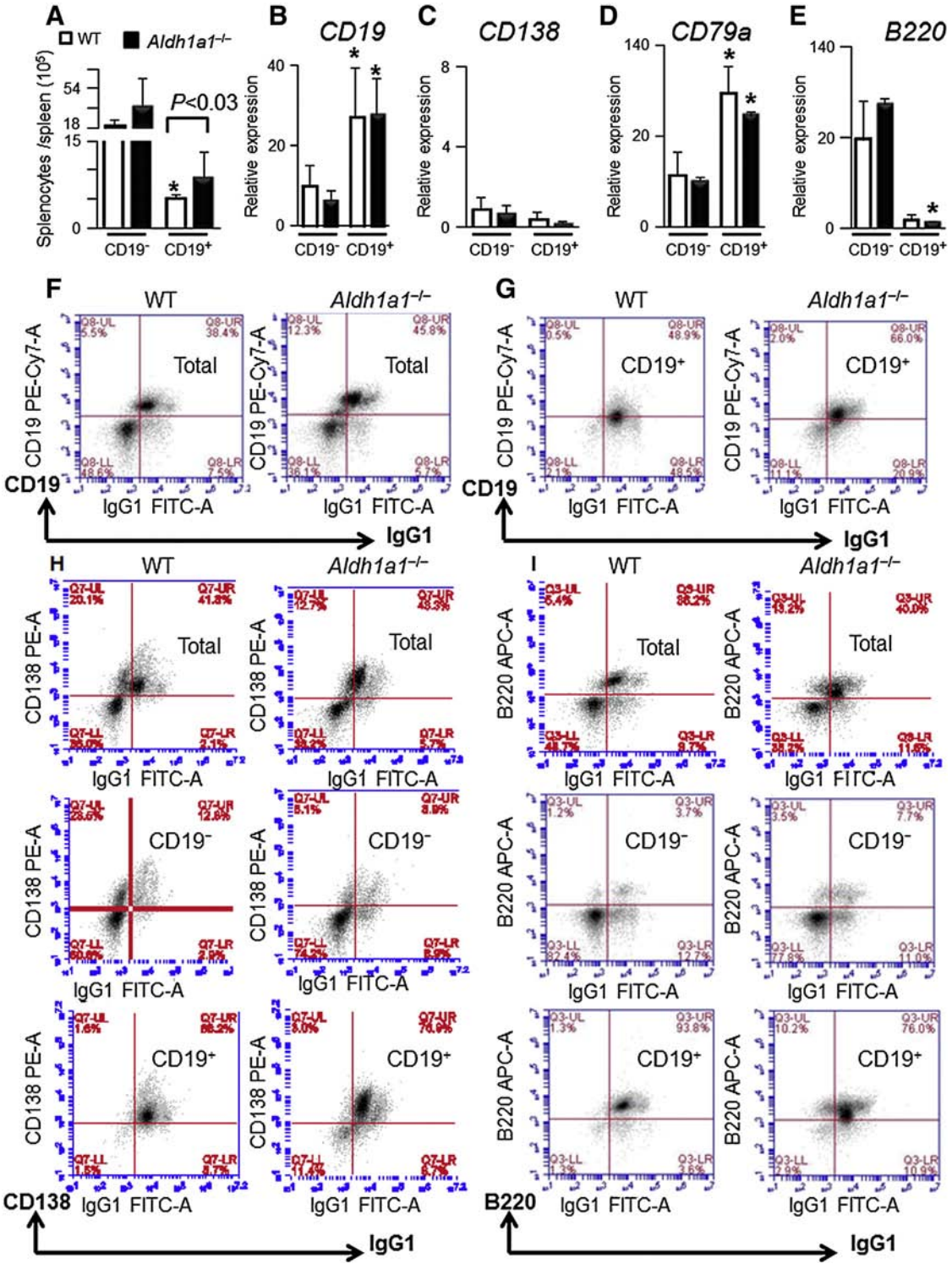


Fig. 2. Increased proportion of CD19$^+$B cells contributes to splenomegaly in *Aldh1a1*$^{-/-}$ mice. (A) CD19$^-$ and CD19$^+$ B cells were isolated from whole spleens of WT and *Aldh1a1*$^{-/-}$ mice using automated magnetic cell separation and double selection with IgG1 and CD19 antibodies (n = 5 per group). Cell populations were quantified. (B–E) Expression of CD19 (B) and plasma markers (CD138) (C) as well as differentiation (CD79a, D) and naive (B220, E) B cell markers were quantified in the isolated CD19$^-$ and CD19$^+$ B cells using TaqMan assays (n = 3 from each group). Data were normalized by *TBP*. Asterisk, significant difference in expression between CD19$^-$ and CD19$^+$ B cells of the same genetic background. Mann–Whitney *U* test. (F–I) Representative immunostaining characteristics (from n = 5 per group) of total splenocytes (F) and isolated CD19$^-$ and CD19$^+$ B cells (G) using FACS analysis. Total splenocytes and isolated CD19$^-$ and CD19$^+$ B cells were simultaneously analyzed with CD19, CD138 (H), and B220 (I) antibodies conjugated with different secondary fluorescent antibodies. For all gates, the percent and total count of all cells staining positive for both antibodies were determined.

transfected with human full length *Aldh1a1* (PCMV6-XL5, OriGene) or empty vector, using the Amaxa Cell Line Nucleofector Kit C (Lonza, NJ). Transient transfections were performed in NIH-3T3 fibroblasts lacking *Pparg* expression using Fugene (Roche, South San Francisco, CA) and the following vectors: HoxA10 luciferase reporter vector (Switchgear Genomics, Menlo Park, CA), control Renilla reporter vector, and murine full length *Rara*, *Pparg*, and *Rxra* constructs according to a previous protocol [16,20].

2.10. Statistical analysis

Oncomine cancer transcriptome database (https://www.oncomine.org) was used as a publicly available platform for data-mining in mRNA-expression studies [30]. The search terms '*Aldh1a1* and multiple myeloma' were used to identify relevant studies with sufficient sample numbers to compare expression of *Aldh1a1* in multiple myeloma and plasma cells in the same dataset. One such dataset was identified [31]. All other data group comparisons were performed using Mann Whitney *U*-test unless otherwise indicated, and correlations were examined by Pearson's test.

3. Results

3.1. Vitamin A metabolism regulates immature B cell populations in the spleen

The topography of RAR activation in mouse spleen was assessed in RARE-lacZ mice treated with and without RA (Fig. 1A). RARE activation in the spleen was heterogeneous and was predominant in the red pulp compared to lymphoid follicles in both non-treated and RA-treated samples.

RA is produced by an ALDH1 family of enzymes ALDH1a1, ALDH1a2, and ALDH1a3. In *Aldh1a1*$^{-/-}$ mice, the spleen is enlarged (256%, Fig. 1B) compared to spleens in WT mice. Spleen architecture is altered in *Aldh1a1*$^{-/-}$ vs. WT mice (Fig. 1C) due to the increased proportion of CD19$^+$ and B220$^+$ B cell populations (Fig. 1D). Germinal center markers MTA3 and BCL6 [32] were expressed at similar levels in *Aldh1a1*$^{-/-}$ and WT spleens, indicating that they were not impaired by *Aldh1a1* deficiency (Fig. 1E). The expression of *Cd1d1* was 61% lower in *Aldh1a1*$^{-/-}$ than in WT splenocytes. The association of splenomegaly with the increase in CD19$^+$ and B220$^+$ B cell populations suggests that differentiation could be impaired in *Aldh1a1*$^{-/-}$ mice.

Differentiated CD19$^+$ B cells are one of the major leukocyte populations in red pulp. Among them, the IgG1$^+$ B cell population was sensitive to RA in pharmacological studies [3,4]. Therefore, we used magnetic cell separation technology to separate IgG1$^+$/CD19$^-$ and IgG1$^+$/CD19$^+$ splenic B cell populations (Fig. 2) to test for effects of *Aldh1a1* deficiency on transcriptional regulation of critical immune pathways in B cells. We termed IgG1$^+$/CD19$^-$ as CD19$^-$ and IgG1$^+$/CD19$^+$ as CD19$^+$ B cells throughout the publication. The splenomegaly seen in *Aldh1a1*$^{-/-}$ mice (Fig. 1B) was associated with an increased number of CD19$^+$ B cells (171%) compared to WT (Fig. 2A). The purity and characteristics of CD19$^+$ B cell population were examined using *CD19* expression (Fig. 2B) and FACS analysis (Fig. 2F–G). Although both cell populations expressed similar low levels of plasma cell marker *CD138* (Fig. 2C), both CD19$^+$ and CD19$^-$ B cell populations were CD138-positive in FACS analysis (Fig. 2H). CD19$^+$ B cells also expressed a mature B cell marker CD79a (Fig. 2D). In contrast, an expression of pro-, mature and activated B cell marker B220 was lower in CD19$^+$ than in CD19$^-$ B cells (Fig. 2E). Both groups were B220 positive in FACS analysis (Fig. 2I). Notably, the expression of all major studied B cell markers was similar in WT and *Aldh1a1*$^{-/-}$ mice. However, *Aldh1a1* deficiency was associated with an increase in the CD19$^+$ B cell population and splenomegaly.

3.2. Dissimilar expression of Aldh1 in CD19$^-$ and CD19$^+$ B cell populations

To investigate whether CD19$^-$ and CD19$^+$ B cells metabolize vitamin A, we examined the expression of enzymes involved in synthesis of Rald (Fig. 3A) and RA (Fig. 3B). The expression of major Rald-generating enzymes (*Rdh10*, *Adh4*) was similar between CD19$^-$ and CD19$^+$ B cells (Fig. 3A, left panel). *Aldh1a1* deficiency moderately decreased *Rdh10* levels (−24%, Fig. 3A, right panel). In contrast, expression of the RA-generating *Aldh1a1* and *Aldh1a2* enzymes was markedly reduced to 6.4% and 18%, respectively, in CD19$^+$ compared to CD19$^-$ B cells (Fig. 3B left panel). *Aldh1a1* was the predominantly expressed member of ALDH1 family of enzymes in both CD19$^-$ and CD19$^+$ B cells (Fig. 3B, left panel). *Aldh1a1* deficiency suppressed the expression of *Aldh1a2* in CD19$^-$ and CD19$^+$ B cells (Fig. 3B, right panel). Thus, CD19$^+$ B cells in *Aldh1a1*$^{-/-}$ mice had reduced levels of all RA-producing enzymes. The change in *Aldh1a1* expression also influenced expression of *Rara*, the primary transcription factor regulated by RA [18]. *Rara* was reduced to 44% in CD19$^+$ vs. CD19$^-$ B cells in WT mice (Fig. 3C, left panel). In *Aldh1a1*$^{-/-}$ CD19$^-$ B cells, *Rara* expression was decreased to 60% compared to WT CD19$^-$ B cells and became similar to that seen in CD19$^+$ B cells (Fig. 3C, right panel).

3.3. Aldh1a1 deficiency results in oncogene expression in CD19$^+$ B cells

To identify the mechanisms altering the number and properties of CD19$^+$ B cells in *Aldh1a1*$^{-/-}$ mice, we analyzed the classic markers of

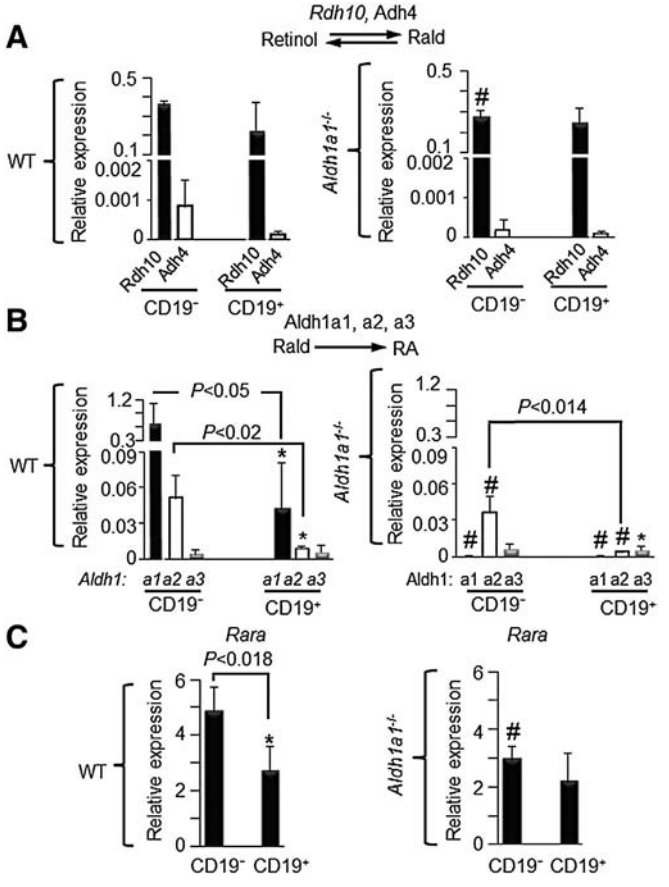


Fig. 3. Diminished expression of RA-generating *Aldh1* enzymes and *Rara* in B cells from *Aldh1a1*$^{-/-}$ vs. WT mice. (A–C) CD19$^-$ and CD19$^+$ B cells (same as in Fig. 2A, n = 5 per group) isolated from WT (left panels) and *Aldh1a1*$^{-/-}$ (right panels) mice were examined for the expression of (A) major retinaldehyde (Rald)-generating enzymes *Rdh10* and Adh4, (B) RA-generating enzymes *Aldh1a1*, *Aldh1a2*, and *Aldh1a3*, and (C) *Rara*. Gene expression was analyzed by TaqMan assays and normalized with *TBP*. *P*, significant difference in expression between CD19$^-$ and CD19$^+$ B cells. Mann–Whitney *U* test (throughout this figure).

B cell apoptosis (caspase 3), proliferation (Mki67) (Fig. 4A), and differentiation (Ebf1 and Pax 5) in all groups (Fig. 4B). The expression of these genes was not altered between *Aldh1a1*$^{-/-}$ vs. WT genotype in the isolated CD19^{-} and CD19^{+} B cells. *Ebf1* and *Pax5* expressions were higher in differentiated CD19^{+} than in CD19^{-} splenocytes; however, these levels were not influenced by WT and *Aldh1a1*$^{-/-}$ genotypes. To identify genes increasing CD19^{+} B cell population in *Aldh1a1*$^{-/-}$ mice, we quantified the expression of 250 inflammatory genes using NanoString Technologies' nCounter System. Gene expression was normalized using six housekeeping genes. The gene cluster analysis revealed that *Aldh1a1* influenced expression of proto-oncogenes (*c-Fos*, *c-Jun*, and *Mafk* kinase) and an opsonin (*C1qb*) (Fig. 4C). *Aldh1a1*$^{-/-}$ CD19^{-} B cells expressed 267% higher levels of *c-Fos* than WT cells (Fig. 3D). These changes were in agreement with decreased expression of *Rara*, a known suppressor of *c-Fos*, in CD19^{-} B cells (Fig. 3C) [33]. *Aldh1a1* deficiency affected CD19^{+} B cells more than CD19^{-} B cells. Specifically *c-Fos* and *c-Jun* expression levels were 711% and 294% higher than those in WT cells (Fig. 4D). This finding was paradoxical because CD19^{-} B cells expressed 15.5-times higher *Aldh1a1* and 5.5-times higher *Aldh1a2* levels than those seen in CD19^{+} B cells from WT mice (Fig. 3B). We hypothesized that another *Aldh1a1*-sensitive suppressor of *c-Fos*/*c-Jun* is active in CD19^{+} B cells.

3.4. Aldh1a1 limits oncogene expression CD19^{+} B cells by a sequential induction of Zfp423 and Pparg

ALDH1 enzymes can induce the transcription factor *Zfp423* which, in turn, controls the expression of the anti-proliferative and anti-inflammatory transcription factor *Pparg* in adipocytes [20]. In splenic B cells, expression of *Aldh1a1* positively correlated with *Zfp423*, specifically in CD19^{+} B cells ($P < 0.001$) (Fig. 5A). *Zfp423* was markedly up-regulated in CD19^{+} (625%) vs. CD19^{-} WT B cells. However, this increase was abolished in *Aldh1a1*$^{-/-}$ CD19^{+} B cells (Fig. 5B), suggesting a regulatory role of *Aldh1a1*. The *Zfp423* expression levels were also

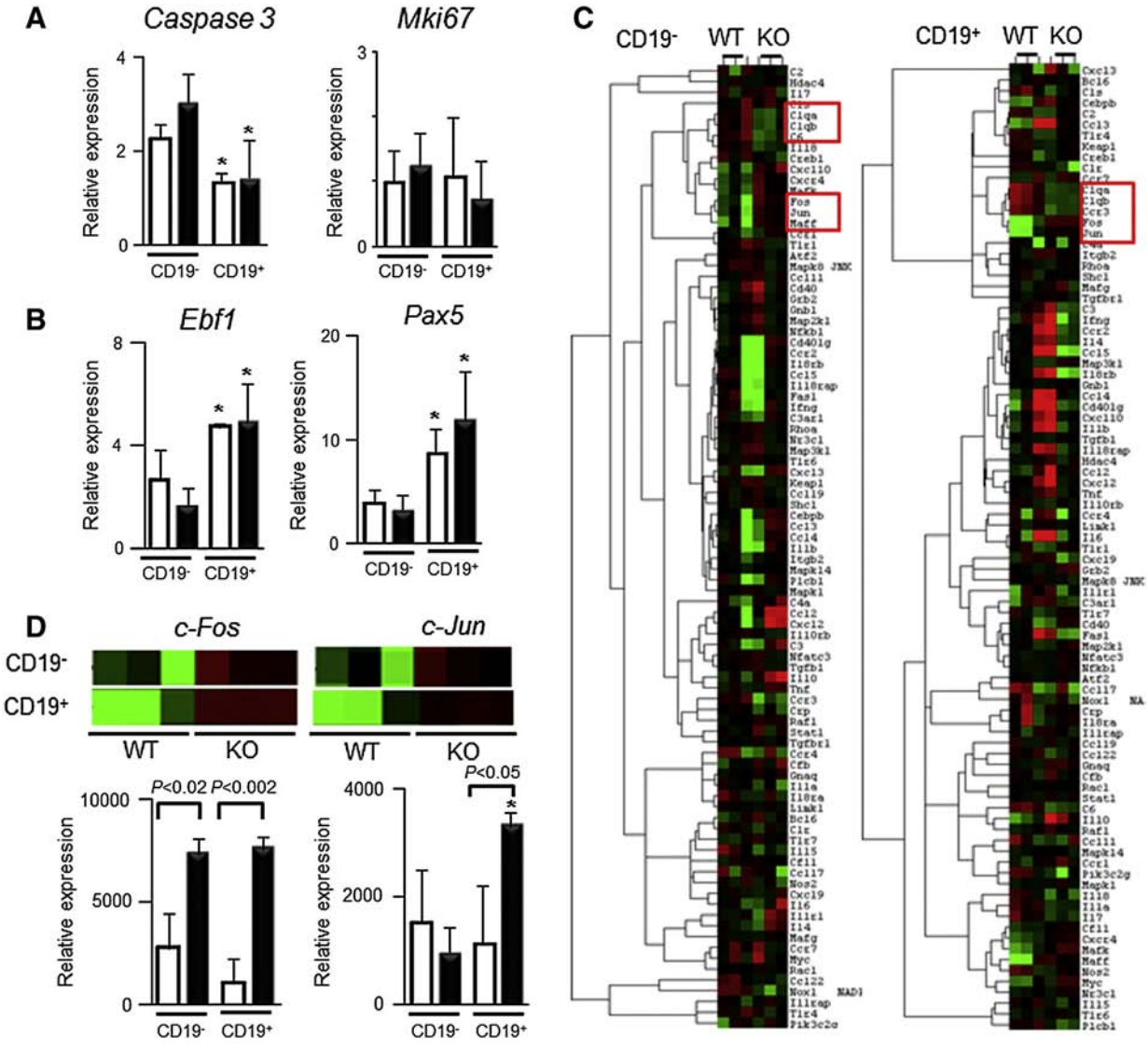
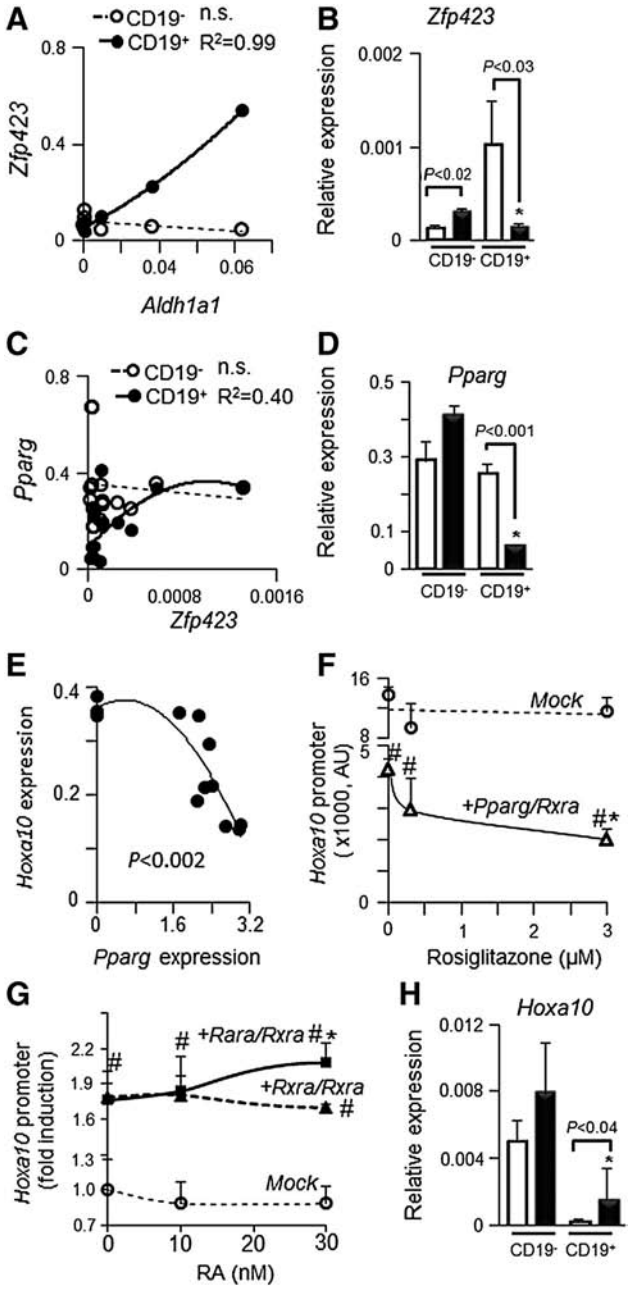


Fig. 4. Increased expression of proto-oncogenic genes in isolated splenic CD19^{-} and CD19^{+} *Aldh1a1*$^{-/-}$ vs. WT B cells. CD19^{-} and CD19^{+} B cells were isolated from spleens of the same WT (white bars) and *Aldh1a1*$^{-/-}$ (black bars) group of mice (Fig. 2, n = 5 per group). (A & B) Expression of apoptosis (caspase3) and proliferation markers (Mki67) (A) as well as B cell differentiation markers (*Ebf1* and *Pax5*) were semi-quantified using TaqMan assays. Data (n = 3 per group) were normalized by *TBP*. Asterisk, significant difference in expression between CD19^{-} and CD19^{+} B cells of the same genetic background. Mann–Whitney *U* test. (C) Selected expression heat maps (red and green colors represent high and low expression levels, respectively) obtained using NanoString Technologies' nCounter mouse inflammation panel. The nCounter GX Mouse Inflammation Kit (NanoString Technologies) consists of 184 inflammation-related genes and six internal reference genes (www.nanostring.com). Red boxes showed statistically significant (n = 3 per group, $P < 0.05$, Mann–Whitney *U* test) clusters of genes. (D) Expression levels of *c-Fos* and *c-Jun*, using NanoString Technologies' nCounter mouse inflammation panel insets show the extracted expression heat maps for *c-Fos* and *c-Jun*. *P*, significant difference between WT and *Aldh1a1*$^{-/-}$ B cells, Mann–Whitney *U* test (n = 3 per group).

correlated with *Pparg* levels in CD19$^+$ B cells (Fig. 5C). In agreement with *Zfp423*'s role in the induction of *Pparg* [20], only CD19$^+$ B cells expressed less *Pparg* (−75%) in *Aldh1a1*$^{-/-}$ than WT splenocytes (Fig. 5D). PPAR response element (PPRE) has been identified in the promoter of transcription factor *Hoxa10* [34], a key inducer of human lymphomyelopoiesis [35]. To examine a possible role for *Pparg* in the regulation of the *Hoxa10* promoter, we performed transfection studies in NIH 3T3 fibroblasts, a cell line lacking endogenous *Pparg* expression. Forced expression of *Pparg* inhibited activation of *Hoxa10* promoter in a gene dose-dependent manner (Fig. 5E). *Pparg* expression also markedly suppressed *Hoxa10* promoter activation in a ligand (rosiglitazone)-dependent manner (Fig. 5F). In contrast, both *Rxra* and *Rara* only moderately activated *Hoxa10* promoter reporter in the presence or absence of RA ligand (Fig. 5G). Consistent with a regulatory role of *Pparg*, CD19$^+$ B cells expressed 400% higher levels of *Hoxa10* in *Aldh1a1*$^{-/-}$ than in WT mice (Fig. 5H). Thus, elevated *Hoxa10* expression in *Aldh1a1*$^{-/-}$ CD19$^+$ B cells could be a direct effect of deficient *Pparg* expression. Other transcriptional mechanisms may also regulate *Hoxa10* in CD19$^-$ B cells. Since elevated expression of proto-oncogenes AP1 (*c-fos*/*c-jun*) and *Hoxa10* is involved in the development of multiple myeloma (MM), we examined the *Aldh1a1* expression in B-cell related cancers.



3.5. RA and/or Aldh1a1 rescues Rara and Zfp423 expressions in myeloma B cell lines

A search of publicly-available cancer gene expression data using Oncomine analysis revealed that B-cell-related cancer cell lines express low levels of *Aldh1a1* compared to other cancer cell lines (Fig. 6A). In our studies in peripheral blood mononuclear cells isolated from healthy donors (Fig. 6B), *Aldh1a1* was a predominantly expressed gene from the ALDH1 family. This pattern of expression was markedly changed in MM cell lines U266B1, RPMI8266, OPM2, L363, and MM1s (Fig. 6B). L363 MM cells expressed no *Aldh1* genes. *Aldh1a1* expression was lower compared to the expression of *Aldh1a2* and *Aldh1a3* in these MM cells. In agreement, the Oncomine analysis findings [31] showed reduced *Aldh1a1* expression in 74 human MM patients, compared to healthy plasma cell controls (Fig. 6C). The RA stimulation of L363 MM cells increased expression of *Znf423*, a human analog of murine *Zfp423* in MM cells (Fig. 6D). *Aldh1a1* overexpression was even more effective in up-regulating suppressors of proto-oncogenes. The expression of a full-length human *Aldh1a1* construct increased *Aldh1a1* expression in U266 MM cells without altering expression of *Aldh1a2* and *Aldh1a3* (Fig. 6E). This overexpression of *Aldh1a1* resulted in an increased expression of both *Rara* (30%) and *Znf423* (500%) in U266 MM cells (Fig. 6F).

4. Discussion

Humoral immune responses are mediated by mature follicular B cells with the help of T cells in splenic germinal centers and, to a minor extent (~10%), by marginal-zone B cells [2]. Cytokines/cytokine receptors, Ig recognition, and antigen presented by APCs, dendritic cells, and/or macrophages can initiate differentiation of B cells and formation of germinal centers to achieve Ig production [11]. In these processes, dietary vitamin A or RA can facilitate differentiation by classic Pax5-dependent pathways in some splenic B cell population [11,12]. Our study revealed a key role for RA-generating ALDH1 enzymes in B cell biology. *Aldh1a1* expression in immature CD19$^+$ B cells and MM B cells is critical for the establishment of a transcriptional profile (Fig.7) that prevents oncogene expression.

Fig. 5. *Aldh1a1* influences expression of *Zfp423* and *Pparg* genes in CD19$^+$ B cells, suppressing promoter activity of *Hoxa10*. (A & B) Correlation (A) between *Aldh1a1* expression (Aldh1a1 expression was shown in Fig. 3B) and expression levels of *Zfp423* (B) in CD19$^-$ (white bars or circles) and CD19$^+$ (black bars or circles) B cells. Gene expression was analyzed in triplicate by TaqMan assays (n = 3 per group). (C & D) Correlation (C) between *Zfp423* expression (B) and expression levels of *Pparg* (D) in CD19$^-$ (white bars or circles) and CD19$^+$ (black bars or circles) B cells (n = 6 in each correlation group). Gene expression was analyzed by TaqMan assays in triplicate. Asterisk, significant difference in expression between CD19$^-$ and CD19$^+$ B cells of the same genetic background; *P*, significant difference between WT and *Aldh1a1*$^{-/-}$ B cells, Mann–Whitney *U* test. (E–G) Promoter analysis of *Hoxa10* in NIH3-3T3 fibroblasts lacking *Pparg* (n = 12). (E) NIH3-3T3 fibroblasts were transiently transfected with full-length *Pparg* overexpression or empty (mock) vector (0). 48 h after transfection the expression of *Hoxa10* was measured by TaqMan assay. *P*, Pearson correlation. (F) Promoter analysis of *Hoxa10* in NIH3-3T3 fibroblasts transiently transfected with mock or full-length *Pparg* overexpression vectors. 24 h after transfection, cells were stimulated with different rosiglitazone concentrations for 15 h (n = 3 in each stimulated group). #, significantly different between cells expressing mock and *Pparg* vector; asterisk, significant difference between *Pparg* expressing cells stimulated with vehicle and rosiglitazone. (G) Cell was transiently transfected with empty vector (Mock) or *Rara* and *Rxra* overexpression vectors (n = 9). 24 h after transfection, cells were stimulated with different retinoic acid (RA, n = 3) concentrations for 27 h. #, significantly different between cells expressing empty vector and *Rara* and/or *Rxra*; asterisk, significant difference between *Rara*/*Rxra* expressing cell stimulated with vehicle (ethanol/DMSO, 50/50%) and RA, Mann–Whitney *U* test. (H) Expression levels of *Hoxa10* in CD19$^-$ (white bars or circles) and CD19$^+$ (black bars or circles) B cells measured by TaqMan assay (n = 3 for each group). Significance was determined as in (D).

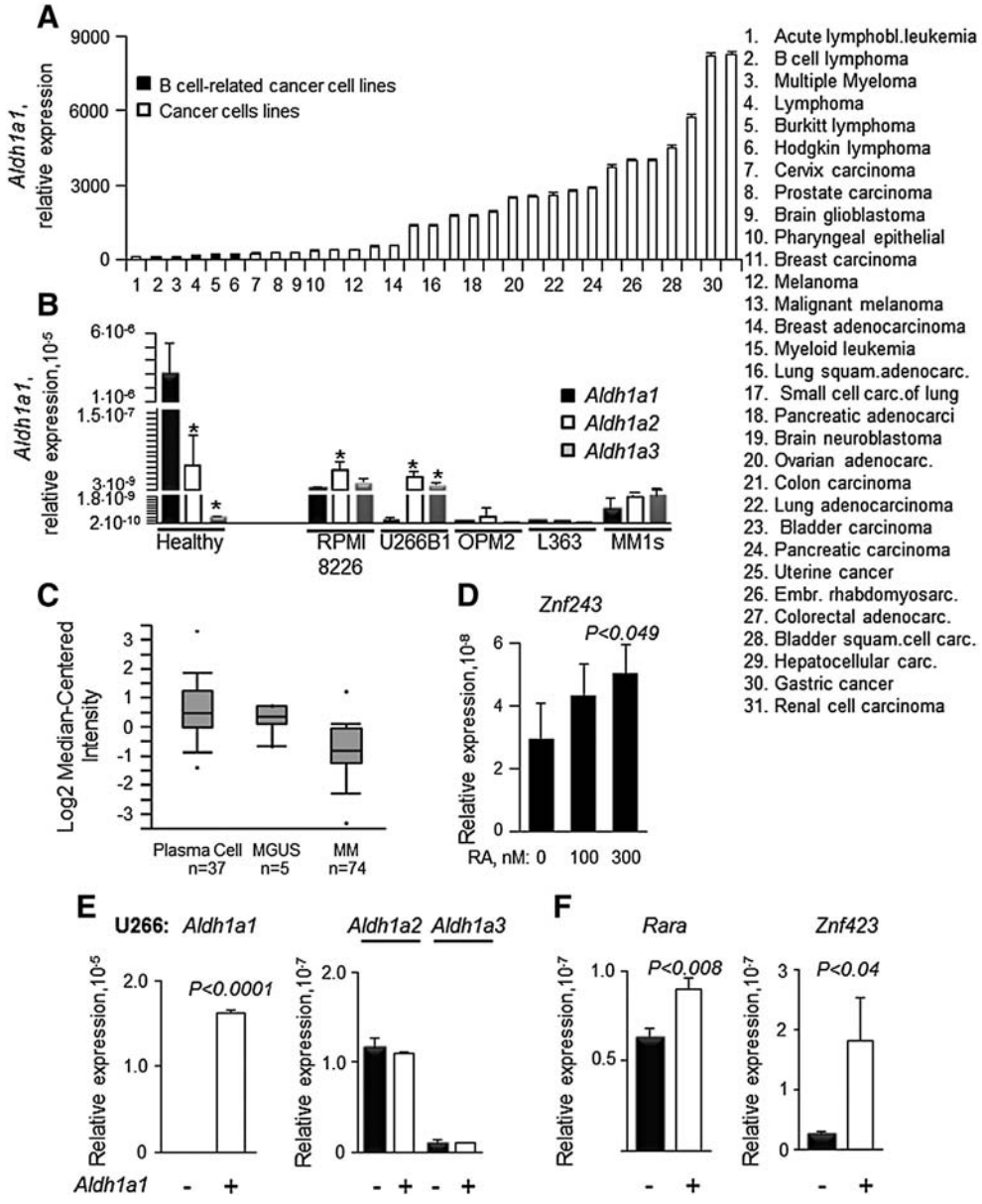


Fig. 6. Impaired *Aldh1a1* expression in human multiple myeloma B cell lines could be rescued by RA or Aldh1a1 overexpression that increased *Rara* and *Znf423* levels. (A) Relative expression of Aldh1a1 in hematological (n = 3) and other cancer cell lines (n = 28). Data were obtained from data mining in Oncomine database and based on the publication by Rhodes et al. [30]. (B) Expression levels of *Aldh1* genes were measured in human PBMC cells (n = 7 donors) and in myeloma cell lines (n = 5): RPMI, U266B1, OPM2, L363, and MM1s (n = 3 per measurement). *P*, significant difference in expression between *Aldh1a1*, *Aldh1a2*, and *Aldh1a3* enzymes within the same cell population. (C) Analysis of *Aldh1a1* gene expression in human multiple myeloma (n = 74), plasma cells (n = 37) and monoclonal gammopathy of undetermined significance (MGUS) (n = 5). Gene expression database analysis (see Materials and methods) identified by Zhan et al. (2002) [31] data shown here (adapted from Oncomine; Rhodes et al. (2007) [30]). The plot boxes are lined at lower, median and upper quartile score values; whiskers extend to 10th and 90th percentiles; dots mark minimum and maximum values. (D) RA treatment of L363 myeloma cell lines increases *Znf423* expression. L363 cells were maintained in RPMI medium containing 1% of UV treated FBS, which is depleted of retinoids. L363 cells were maintained in this medium 24 h prior to RA stimulation and during treatment with RA. *Znf243* expression was measured 48 h after RA treatment (n = 3). *P*, significant difference, Mann–Whitney *U* test. (E) Expression levels of *Aldh1a1* (left panel) and *Aldh1a2* and *Aldh1a3* (black and white bars in the right panel) in U266B1 cells transiently transfected with empty (−) or human full length Aldh1a1 overexpression plasmid (+) (n = 3 independent experiments). Expression levels were measured in triplicate using TaqMan assays 24 h after transfection. *P*, significant difference, Mann–Whitney *U* test. (F) Expression of *Rara* (left panel) and *Znf*423 (human analog of mouse*Zfp423*, right panel) in U266B1 (black bar) transfected with empty (−) and *Aldh1a1* overexpression vector (+) (n = 3 independent experiments). *P*, significant difference, Mann–Whitney *U* test.

Aldh1a1 expression was consistently predominant over other RA-generating enzymes from the ALDH1 family in healthy B cells in mice and humans (Figs. 3B and 6B). *Aldh1a1*-dependent pathways in isolated B cell populations were different from those altered by administration of RA or manipulation of dietary vitamin A content. RA administration has two major sites of action related to B cell functions. It improves antigen presentation and IgA production at mucosal sites [15] and induces proliferation and differentiation of $IgG1^+$ splenocytes in germinal centers [3,4,11]. In these scenarios, endogenous RA was produced by APCs and stimulated final B cell differentiation in germinal centers [36]. We showed that under physiological conditions, intense endogenous RAR activity was associated with red pulp (Fig. 1A). In agreement, we found the highest *Aldh1a1* and *Rara* expression levels in $IgG1^+CD19^-$ ($CD19^-$) B cell populations (Figs. 2, 3). $CD19^-$ is a potentially heterogeneous population comprised of naive and mature $B220^+$ B cell populations. Both *Rara* and *Aldh1* expressions were decreased in the $IgG1^+CD19^+$ ($CD19^+$) population. This loss of endogenous RA production in $CD19^+$ B cells could later allow them to receive a paracrine signaling of RA-producing APCs after they enter germinal centers. The physiological expression of *Aldh1* genes in $CD19^+$ vs. $CD19^-$ B cells in WT mice

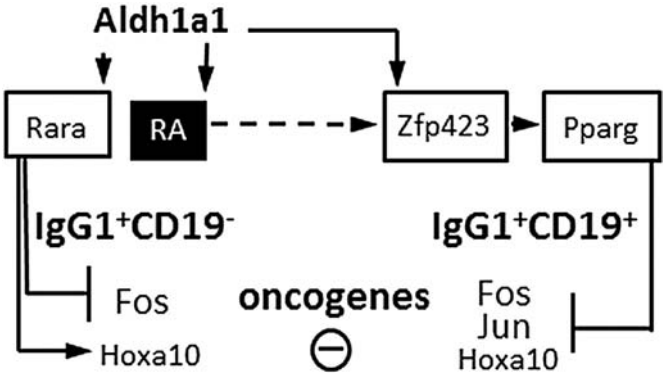


Fig. 7. Schematic diagram of the *Aldh1a1* dependent pathways in CD19$^-$ and CD19$^+$ IgG1$^+$ B cell populations in the spleen. *Aldh1a1* is expressed at higher levels in CD19$^-$ vs. CD19$^+$ B cells. *Aldh1a1* also shapes the expression of transcription factors in CD19$^+$ population, where it is responsible for the induction of *Zfp423* and *Pparg*. Expression of *Pparg* suppressed proto-oncogenes *c-Fos*, *c-Jun*, and *Hoxa10*.

prevented increase in expression of oncogenes *c-Fos*/*c-Jun* and *Hoxa10* (Figs. 4D, 5H), due to the *Aldh1a1*-dependent induction of oncogene suppressors (*Zfp423 and Pparg*) in these cells (Fig. 5B, D). The disruption of *Aldh1a1* regulation in *Aldh1a1*$^{-/-}$ mice markedly compromised B cell oncogene profiles during differentiation (Figs. 4, 5), increased red pulp proportions, and reduced expression of *Cd1d1* that is involved in interactions between T and B cells (Fig. 1D, right panel).

Classic CD19$^-$ and CD19$^+$ B cell differentiation via *Ebf1* and *Pax5* [37] appear to be not impaired in the absence of *Aldh1a1* (Fig. 4B). The breakthrough in the understanding of the mechanism increasing CD19$^+$ population came from the NanoString analysis (Fig. 4C). *Aldh1a1* deficient CD19$^+$ B cells expressed proto-oncogenes *c-Fos*/*c-Jun* forming the transcription factor AP1. Elevated *c-Fos*/*c-Jun* and also *Hoxa10* expressions are distinct features of B cell-dependent hematological neoplasms, including lymphomas and multiple myeloma [38–40]. *Hoxa10* overexpression in hematopoietic cells is sufficient to impair murine and human lymphomyelopoiesis and leads to acute myeloid leukemia [35,41]. Notably, all five human multiple myeloma (MM) cell lines expressed 100-times less *Aldh1a1* than normal PBMC cells (Fig. 6B). Similar findings in B cell cancers and in plasma cells from MM patients were available from other studies [31] that we identified through database analysis (Fig. 6A, C). The major RA-generating enzyme in these cancer cells was *Aldh1a2* suggesting that the loss of *Aldh1a1* contributed to B cell neoplastic pathology. Previous studies showed that only combined treatment of RA and rosiglitazone can induce U266 differentiation [14]. Our data provide a mechanism and rationale for the treatment of MM B cells lacking *Aldh1a1* with RAR and PPARγ agonists.

Increased *c-Fos* expression in *Aldh1a1*$^{-/-}$ CD19$^-$ B cells appears to be consistent with the known competitive relation between AP1 and RARα activated by RA [33]. Indeed, *Aldh1a1*$^{-/-}$ CD19$^-$ B cells had reduced expression of both *Aldh1a1* and *Rara* (Fig. 3). Forced expression of *Aldh1a1* in U266 cells can readily increase *Rara* expression (Fig. 6). An unexpected result of our study was the finding of more profound transcriptional changes in CD19$^+$ compared to CD19$^-$ B cells expressing markedly less *Aldh1a1* in WT mice (Fig. 3B). WTCD19$^+$ cells expressed higher levels of proto-oncogenes *c-Fos*, *c-Jun*, and *Hoxa10* than *Aldh1a1*$^{-/-}$ CD19$^+$ B cells. Increased number of CD19$^+$ cells contributed partially to the splenomegaly in *Aldh1a1*$^{-/-}$ mice. This phenomenon could be based on *Aldh1a1*-mediated changes on the transcriptome. During adipogenesis, *Aldh1a1* induces expression of the transcription factor *Zfp423*, which in turn induces *Pparg* [20,42]. Previous investigations highlighted competition between *Pparg* and *c-Fos*/*c-Jun* without engaging PPRE [43,44]. PPRE response element was found in the *Hoxa10* promoter [34]. Our studies connected *Aldh1a1* to the regulation of all these transcription factors in B cells (Fig.7). *Aldh1a1* did not support *Zfp423* expression in CD19$^-$ B cells probably due to the specific transcriptional environment. It is possible that high *Aldh1a1* levels in CD19$^-$ B cells produced RA for paracrine signaling that induced *Zfp423* in CD19$^+$ B cells. These regulatory mechanisms remain to be investigated in the future. We found that *Aldh1a1* is required for the *Zfp423* induction in differentiating CD19$^+$ population (Fig. 5B). *Zfp423* and *Pparg* levels were higher in CD19$^+$ B cells in WT compared to *Aldh1a1*$^{-/-}$ mice. This link was suggested by a significant correlation in CD19$^+$ B cells (Fig. 5C). The causative link between *Aldh1a1* and *Zfp423* expressions was demonstrated in human U266 MM cells. *Aldh1a1* expression rescued *Znf423* (human analog of mouse *Zfp423*) in U266 MM cells (Fig. 6F). RA treatment of MM cells can also rescue *Znf423* expression (Fig. 6D), suggesting that ALDH1a1 acts in part via autocrine RA generation. Since *Pparg* is induced by *Znf423*, the short (24 h) transfection period was not sufficient to also observe significant increase in *Pparg* expression. However, the relationship between *Zfp423* and *Pparg* has been widely documented [20,42]. *Pparg* induction by *Aldh1a1* appears to be a critical event, because *Pparg* was an effective suppressor of *Hoxa10*, a key transcription factor perturbing myeloid and lymphoid differentiation in mice and humans [34,35,41]. *Pparg* inhibited the promoter of *Hoxa10*, while *Rara* and *Rxra* only modestly regulated this transcription factor (Fig. 5F, G). Consequently, *Hoxa10* was upregulated in *Aldh1a1*$^{-/-}$ CD19$^+$ B cells expressing less *Pparg*. Studies investigating the effects of vitamin A-deficient diets reported splenomegaly and an increase in plasma IgG1 levels in mouse models of autoimmune disorders [45], whereas the production of specific IgG1 antibodies in the immunized mice was impaired [46]. Multiple mechanisms have been proposed to explain these phenomena, including RA-dependent production of IFNγ from T cells [47] and dendritic CD103$^+$ cell subsets [48], but the role of B cells was unclear. Our data highlight an autocrine and/or paracrine ALDH1 function in differentiating B cells that regulates two key transcriptional oncogene suppressors *Rara* and *Pparg*. This suggests a gene–environment paradigm for early-stage deregulation of oncogene profiles in IgG1$^+$ B cell subsets through compromised vitamin A metabolism.

5. Conclusion

Our findings showed the critical role of the retinoic acid-generating ALDH1a1 enzyme in the sequential induction of oncogene suppressors *Rara* in IgG1$^+$/CD19$^-$ B cells and *Zfp423*/*Pparg* in IgG1$^+$/CD19$^+$ B cells during B cell differentiation. In the absence of these suppressors, B cells acquire oncogene *Ap1* and *Hoxa10* expressions that lead to IgG1$^+$/CD19$^+$ B cell expansion and splenomegaly. Reduced expression of *Aldh1a1* and oncogene suppressors *Rara*, *Zfp423*, and *Pparg* is a characteristic property of malignant human multiple myeloma B cells. Importantly, ectopic expression of *Aldh1a1* or RA effectively rescues *Rara* and/or *Zfp423* expression. The understanding of the role of ALDH1a1 in B cell differentiation can shed light on the early stages in the development of malignant hematological disorders and may lead to the development of novel therapeutics.

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

The project during the past year accelerated when the CRADA's were executed. In the first two years, we optimized the primary genotyping and molecular methods, and the follow-on validation methods. We also expanded the capabilities of our highly-flexible DAPER database and software tools in the present reporting year. To digitize the pathological records, we initiated the high through-put software customization (ABBYY FlexiCapture) for analysis of 1829 longitudinal veterinary records and AFIP/JPC pathological records. In addition, we have initiated the DoD military dog pathology reports to identify cancer bearing dogs for cancer classification and selection of cases and controls. In the first year we invented an entirely novel approach to conducting genome wide genetic association (GWA) analysis – genomewide IUT analysis (GIA); and in the second year we further validated it. In this second reporting year, we integrated IUT and Bootstrapping as an additional innovation with outstanding utility. Dr. Alvarez's presentation of these methods and results to leaders in the fields of genetics and canine genetics resulted in uniformly positive feedback from them (and multiple requests for collaboration). In addition, Dr. Huang has developed the LDPMap algorithm for enabling accurate query of biomedical terms in the database. In addition, we co-authored (Alvarez) a published study that was not based on the present military dog project, but which made use of the same data mining and analysis methods that will be used in our study. The LDPMap algorithm paper is accepted to BMC Medical Genomics. Dr. Rowell, one of our investigators (originally as a predoctoral student), moved on to conduct a postdoctoral fellowship with a pre-eminent dog geneticist at NIH and, after only a year there, is being recruited for a tenure track faculty position at OSU. Dr. Rybaczyk, another of our investigators (originally a postdoctoral fellow and promoted to research scientist) went on to be an NIH T32 Fellow at MSU, which is essentially a pre-faculty position. Dr. Alvarez was promoted to Associate Professor with tenure by OSU and is now under consideration for leadership training in the OSU College of Medicine.

15. SUBJECT TERMS

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Introduction

The purpose of this proposal is to provide insight into gene environment interactions. It leverages the simplified genetics and detailed records of the military working dog population. There are several critical aspects to meeting the aims of this proposal. 1) development of data driven selection criteria, 2) biological sampling of representative dogs, and 3) generation of mathematical methodologies capable of handling heterogenous data and statistical tests in consistent manner and providing clear and understandable results that are biologically valid. Here we provide a breakdown of the previous year's work and document our progress towards achieving the specific aims we proposed. While the overall progress of this project is summarized in the Annual Report by Dr. Carlos Alveraz (Lead PI from NCHRI), here are the tasks in which I (Huang from OSU) have engaged in.

Body

Task 1- Regulatory Approval:

i) Cooperative Research And Development Agreements (CRADAs): Both the data and biological CRADAs between Nationwide Children's Hospital (NCHRI; Alvarez, Lead PI, home institution)/OSU (Huang and Couto, Partnering PI's) and DoD/USA were executed by 2013.

ii) Animal use approval (Institutional Animal Care and Use Committee, IACUC): The animal hospital at Lackland AFB received AAALAC accreditation that is mandatory for military IACUC approvals in 2012. In 2013, we submitted final revisions on our IACUC protocol for the collection of biological samples and Lackland veterinary approval was granted; and final Lackland AFB oversight approval was granted and those documents were submitted to DoD CDMRP grant administration. Currently, there is one final approval from ACURO pending (and expected, according to their original anticipated timeline, within ~1 month), at which time biological sample collection can be initiated.

Task 2- Data Capture of Veterinary Records: By having Ms. Michelle Perez, Veterinary Technician, embedded in the military dog health service at Lackland AFB, we have been acquiring clinical and associated data from military dogs. This was made possible by the execution CRADA's in 2013 (Task 1). The veterinary clinical cancer and medical records expertise was provided by Dr. Couto. We have been using that data in two parallel tracks. (i) In the first track, we have been using data forms to create advanced methods for capturing paper-based data and converting those to electronic data (which is classified as raw or manually confirmed to accurately represent the original) (using custom form versions of ABBYY software). That work was initiated in the technical sense before we had CRADA's in place to use it on real DoD military dog health records. In 2013, Mr. Terry Camerlengo and his subsequent replacement Mr. Jacob Aaronson (under supervision of Drs. Alvarez and Huang) worked with actual military dog health records (scanned by Vet. Tech. Ms. Perez at Lackland AFB) to create those custom electronic versions of paper forms. Specifically, they initiated the development of custom scanning and data capture from DoD military dog health record form 1829 (which are generated for each health visit, providing longitudinal data) and from AFIP/JPC pathology reports (which are generated for essentially all diagnostic cancer biopsies and sometimes for necropsy). That required significant efforts from ABBYY support and Research IT, NCHRI to implement. This effort is ongoing. If one or both final customized forms are successful in the near future, we will be able to scan any future records and automatically isolate each 1829 and pathology report. Importantly, we would also be able to scan the many prioritized full records scanned and archived in our database in "track ii". (ii) In the second track that was initiated in 2012 and is ongoing through 2013, we have used different indicators to prioritize individual dogs that are particularly important to our study and have begun scanning their complete records (except for some associated clinical test data that could not be scanned – e.g., EKG's on thin perforated paper (which would have risked their destruction in our portable automatic-feed scanner). We are mainly focused on dogs that have had cancer or most likely would have had it by now if they had high risk (according to age). We thus acquired a list of all Lackland AFB dog health records for which there are AFIP/JPC pathology reports. This was made possible by our primary military dog program contact, LTC Cyle Richard. He provided us that list, which he received from AFIP/JPC; in this way, we did not have to review thousands of records to identify those that contained pathology reports or cancer diagnoses. This in turn allowed us to examine DoD military dog puppy program dog (DoD bred dogs vs. purchased dogs) pedigrees for selection of affected and unaffected littermates or half siblings. From this analysis we identified a relatively small number of popular breeders that had many litters with different partners.

Task 3-Methodolgy Development:

Task 3 is advanced about as far as the data types we have acquired to date. Once final IACUC approval is granted (expected within the month) and we begin to acquire military dog samples after, we expect to be able to deploy the methodologies we have developed. Specifically, we have validated the principal new methods using data from previously-acquired Greyhound osteosarcoma case and control samples, and from data published by the LUPA Consortium (Vaysse A et al. 2011. Identification of genomic regions associated with phenotypic variation between dog breeds using selection mapping. PLoS Genet. 7(10):e1002316. PubMed PMID: 22022279).

In the first year's Annual Report, we included two manuscripts (Rybaczyk et al. and Rowell et al.) that used a new methodology we developed under the present program. Both those manuscripts were submitted for publication in leading genetics journals, and we have been addressing reviewers criticisms and advice. Throughout 2013, we continued to refine and validate those studies. Specifically, this work involves the invention of entirely novel techniques to conduct genomewide association analysis or GWAS (Balding 2006) and multidimensional statistical analysis: Intersection Union

Testing or IUT (Berger 1982; Berger 1997) combined with Bootstrapping (both well established, but the approach has never been used for these applications).

The original focus of these works was on development of the IUT. In the course of improving the methods to address reviewer comments during this reporting year, we determined that the integration of Bootstrapping with IUT is a major innovation and advantage (Fig. 1). The greatest concern about our manuscripts was that the IUT method does not generate conventional measures of statistical significance (p-values), despite the fact that the method empirically ranked IUT-"significant" hits correctly (according to detection of true positives in published datasets). [Notably, that is the major focus of applications of IUT to biology and high throughput gene expression data. Some have proposed solving it using Bayesian approaches, but after many years, no one has had success doing so.] By adding Bootstrapping upstream of IUT, we are able to give another type of measure of robustness of results – a confidence (vs. significance) measure (Bootstrap Confidence Value, BCV).

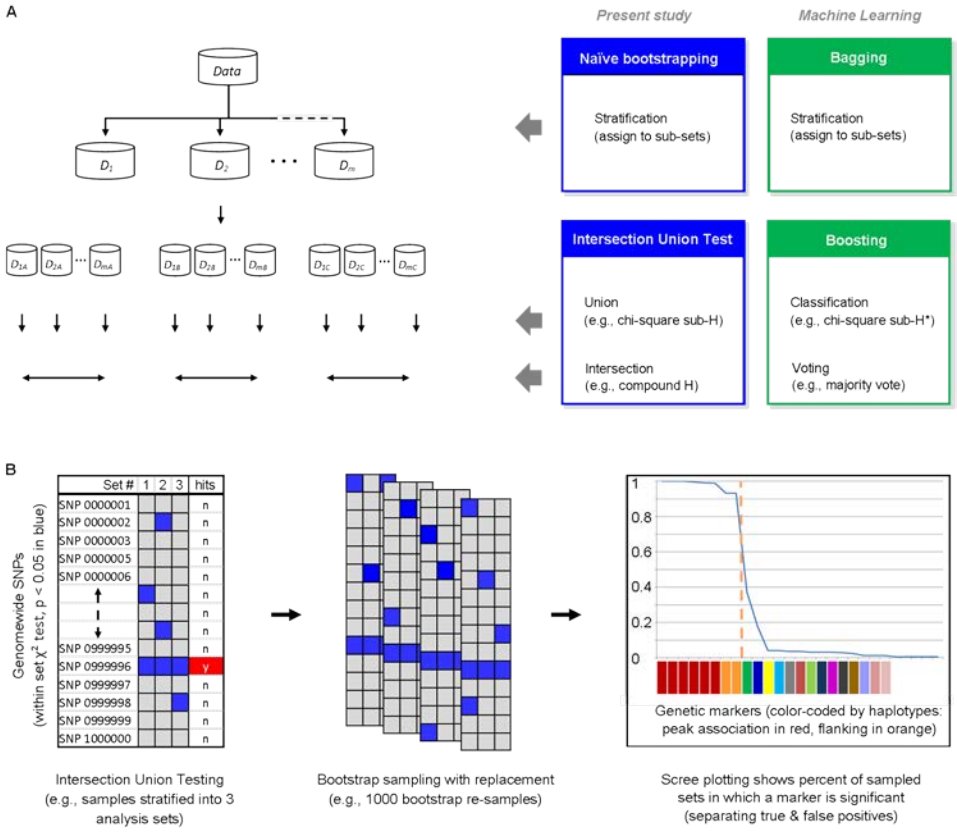

Figure 1. Schematic of integrated Bootstrapping and Intersection Union Testing (IUT) for genetic analysis. (***A***) The schematic on the left shows how a single dataset is repeatedly subsampled (with replacement) and each subsample of cases and controls is then put through the IUT compound hypothesis: i) for each subset of an IUT group, which genetic markers have statistically significant frequency differences in cases and controls, ii) keep only the markers that are significant in all subsets of an IUT (thus not requiring multiple testing correction). Right hand notations compare our methods, which are considered hypothesis tests, to analogous approaches in the field of Machine Learning, which are not considered hypothesis tests but rather learning or predicting. (***B***) Illustration of how IUT works in first panel: each marker (SNP) is tested for significance in each subset of an IUT group (set #1, 2, 3); only those significant in all are kept. Second panel illustrates how repeating the process on 1000 Bootstrap replicates (4 shown) can be used to plot the proportion of times a marker is positive in the 1000 (scree plot, third panel).

In this reporting year we discovered strong evidence that our method is very sensitive and specific based on analysis of the genetic contributions to the complex trait of dog size as a test (using the Vaysse et al. dataset cited above). Specifically, we reanalyzed that published data and, not only identified those authors' two genomewide significant hits using conventional methods, but we also found additional IUT-genomewide significant hits that they missed (but which have been shown to be true positives in other canine genetics studies). We also generated new evidence that Bootstrap/IUT methods i) have increased ability to detect weak signal (a critical need for complex genetics such as cancer risk) and ii) does not require correction for population structure when the analysis is designed properly. We did this by analyzing the most complex dog trait reported by Vaysse et al (ref. above) – sociability (the response of a dog when approached by another dog or a human) as a test (experimental support for these claims were provided in figures within the Q7 and Q8 Quarterly Reports).

In addition to the genetic analysis, we also face the challenge of enabling effective query of medical terms once the database is completed. Given the large collection of biomedical term resources such as ICD9, ICD10, and SNOWMED-CT for clinical diagnosis, Gene Ontology for gene information, and other drug databases, different naming systems can significantly affect the search accuracy. In a collaboration with Dr. Yang Xiang (OSU Biomedical Informatics), we tackle this issue by using the Unified Medical Language System (UMLS) developed by the National Library of Medicine (NLM) of NIH. UMLS has a hierarchical structure for the medical vocabularies collected from more than 100 databases including the ones mentioned above. Each biomedical term is given a unique ID. In order to map the user input words to the exact biomedical terms and IDs in any query, NLM provides a set of tools called Metathesaurus

Browser and MetaMap. However, these tools are quite strict on the input term and often fail if the input term is contains small errors or even small discrepancy with the target term. So we developed a new algorithm called layered dynamic programming mapping (LDPMap) and it provides much higher accuracy in mapping the query terms to the target medical terms. The algorithm was presented in the International Conference on Translational Bioinformatics in Seoul, Korea, in October 2013 and the manuscript was accepted to the special issue for BMC Medical Genomics to be published in 2014 (Ren 2014).

Task 6- Adaptation of existing resources, data storage and hosting:

We have a secure virtual machine called Research DAPER or resdaper developed initially by Mr. Camerlengo and continued by his replacement Mr. Aaronson (supervised by Drs. Alvarez and Huang). The machine exists on the secure NCHRI (Alvarez) network behind a firewall. It can only be accessed by highly-secure VPN using two factor authentication. We have an instance Microsoft SQL Server stored on the machine. Microsoft SQL Server is an industry-leading relational database product that we use to store all of our documents after they have been digitalized. With a relational database, you can quickly compare information because of the arrangement of data in columns. The relational database model takes advantage of this uniformity to build completely new tables out of required information from existing tables. In other words, it uses the relationship of similar data to increase the speed and versatility of the database. The "relational" part of the name comes into play because of mathematical relations. Each table contains a column or columns that other tables can key on to gather information from that table. We have many fields that we can filter and sort on that we can use to retrieve items. Ultimately, this will include all clinical and associated data, environmental data and genetic (genotype), epigenetic, and genomic/molecular (phenotype) data. The user interface is under construction. We will have a web user interface that can be accessed by those with secure credentials. We have used Microsoft asp.net MVC to build the user interface. Using the model view controller pattern gives us the benefit of separating the representation of information from the user's interaction with it .The model consists of application data, business rules, logic, and functions. A view can be any output representation of data, such as a chart or a diagram. The controller mediates input, converting it to commands for the model or view.

In Task 2(i) we discussed the conversion of paper health records to digital versions using ABBYY software – mainly the 1829 form and the AFIP/JPC pathology reports. That digitized data will be fully accessible and searchable through the web interface mentioned above. In addition, the Task 2(ii) scanned complete veterinary clinical records will be directly linked as PDF format. This will allow analysis of digitized data with the option of follow-up detailed analysis of full health records on the same database/tools ensemble “resdaper” (or confirmation/cross-validation of critical data). We have thus installed the ABBYY FlexiCapture software and all of the components which include The Processing Server. That is the server that controls the operation of the Processing Stations. We installed the Licensing Server, the server that stores and manages licenses. We installed the Application Server, the server that controls the operation of the other components. We installed the Application Server components, which will allow operators to connect to the server and work using a web-browser. We also have the Application Server component which allows operators of web stations to register with the system and create requests for access rights to the web station. It provides operators of web stations with a single entry point into the system.

Task 7: Pathway analysis and functional characterization.

Task 7a is complete. I (Alvarez) have been conducting extensive data mining and analysis that are honing those skills which will ultimately be applied to the study of cancer in military dogs. That includes work on osteosarcoma risk candidate genes from Greyhounds (to be published in Rowell et al. manuscript mentioned above) and LUPA candidate genes for multiple canine traits (also discussed above). Most importantly, the Greyhound study implicated small genomic regions with one or two genes each. This allowed use of human cancer data and analysis servers to predict which were likely to be cancer genes and whether the human evidence suggested the cancer risk gene variant was likely to result in up or down regulation. For example, the IntoGen server permits analysis of gene expression and genome alterations associated with diverse cancer types. But other analysis servers, such as NextBio, Oncomine, KMplot and BioGPS provide different tools to mine the same gene expression data in very different ways. For example NextBio make meta-analysis of any subset of studies and KMplot generates Kaplan Meier survival plots for a subset of cancer types that have very large numbers of data available. With this data in hand, it is possible to generate hypotheses and to conduct cross-validation studies. For example, in the Greyhound osteosarcoma case, we can test those predictions by analyzing genetic association candidates in a canine osteosarcoma tumor gene expression dataset which includes Greyhound, Golden Retrievers, Rottweiler’s and mixed breed dogs. Because there are orders of magnitude more human data than canine, it is critical to be able to make use of it.

Among the major aspects of genetic/genomic studies are contextualization according to biochemical or genetic pathways, cross-dimensional/platform validation, and comparative genomics/cross-species validation. To that end, I have

conducted studies in these aspects of cancer genetics. Among those, I mined for genetic evidence that the enzyme aldehyde dehydrogenase is involved in multiple myeloma (for which there is experimental evidence generated by a collaborator studying this with their own funding). As a result of the latter analysis, my analyses were added to a manuscript that was recently accepted for publication. Although the following work was not based on our military dog data, my contributions involve the same analyses that will be conducted with canine cancer candidate genes: Yasmeen R., Meyers J. M., Alvarez C. E., Thomas J. L., Bonnegarde-Bernard A., Alder H., Papenfuss T. L., Benson D. M. Jr, Boyaka P. N., Ziouzenkova O. (2013) Aldehyde dehydrogenase-1a1 induces oncogene suppressor genes in B cell populations. Biochim Biophys Acta 1833:3218–3227. (See Appendix II) For example, I conducted the analysis shown in Figs. 6A and 6C. That critical information shows that the biology suggested by the Yasmeen et al. molecular/biochemical study can be cross-validated by public datasets involving other types of evidence (here gene expression). Similarly, we expect that the vast data available on human cancers will yield supporting evidence for canine cancer findings from the project that is the subject of this report.

Task 8- Project management, Quality control and assurance, and Security:

The most important change in this reporting year is the execution of the CRADA's which allowed us to acquire DoD military dog data. We established a footprint at Lackland and implemented security protocols in accordance with our agreements. We are conducting quality control evaluations for our data collection techniques to assure that we are collecting appropriate data. Once we have assured high quality data we will begin automated import into the database. We are also cross-validating medical and pathology records to assure accurate diagnosis. We initiated collaborations with Dr. David Gutman at Emory University and hope to use his automated pathology data base to facilitate confirmation of sample classification.

As of June 1st, 2013, Task 8 duties attributed to Dr. Rybaczyk (who has moved on in his academic career, as an NIH T32 Fellow, Michigan State U.) are being done by Dr. Alvarez. This transition was been smooth. A job listing was posted for a replacement postdoctoral fellow. Dr. Alvarez interviewed a highly-qualified postdoctoral fellow named Dr. Sohan Lal (currently postdoctoral fellow at Yale), but unfortunately Dr. Lal was forced to accept another position at Yale due to imminent expiration of his visa status. There is another candidate under consideration; the goal is to hire that person prior to initiating the biological sample collection.

The replacement for Mr. Camerlengo – computer programmer – was a success. His role has been taken up by Mr. Jacob Aaronson, who may not be as experienced as Mr. Camerlengo but appears to have greater affinity for the biomedical aspects of computational sciences. In particular, he is a research staff in the Informatics Research & Development Team of the OSU Department of Biomedical Informatics and has extensive experience in developing databases and webtools/interfaces for biomedical applications in the Medical Center. Mr. Aaronson quickly completed his NCHRI orientation, security clearance/ID badge, and vaccination requirements. Most importantly, he rapidly oriented himself in the project and is performing high quality work.

Key Research Accomplishments

- Execution of institutional agreements (CRADA's) between NCHRI (Alvarez)/OSU (Huang, Couto)
- Completion of all facets of IACUC between NCHRI and Lackland AFB through final Lackland AFB oversight approval (currently waiting for final ACURO approval expected within ~1 month)
- Successful embedding of NCHRI (Alvarez) Veterinary Technician, Ms. Michelle Perez within the military dog health service at Lackland AFB
- Successful scanning of veterinary clinical records by Ms. Perez at Lackland AFB, transmission of encrypted data to NCHRI, and uploading to DAPER database
- Continued development and validation of a scale free, high-power statistical methodology capable of resolving signal from noise in high throughput genetic/genomic data (IUT/GIA) by incorporation of Bootstrapping
- GIA manuscripts continue to be refined since receiving comments from peer reviewers
- GIA grant application to NIH is being refined based on peer reviewer critiques
- Expansion of our highly flexible data-infrastructure that is robust enough to handle military working dog records and queries of said records
- Initiation of high through-put software customization (ABBYY FlexiCapture) for analysis of 1829 longitudinal veterinary records and AFIP/JPC pathological records
- Initiation of DoD military dog pathology reports to identify cancer bearing dogs for cancer classification and selection of cases and controls

- Initiation of DoD military dog “puppy program” pedigree analysis for identification of high and low cancer risk lineages
- Development of LDPMap algorithm for mapping query terms to the exact biomedical terms in UMLS.

Reportable Outcomes

- Dr. Jennie Rowell, having received her PhD from OSU for her work at NCHRI (Alvarez), joined the lab of one of two pre-eminent dog geneticists in the world, Elaine Ostrander, NIH, as postdoctoral fellow. The first week of Nov. 2013, she has a job interview for a tenure track position at the College of Nursing, OSU
- Expansion of DAPER database capabilities maintaining strong security
- Mr. Terry Camerlengo moved from OSU to the Battelle Institute as a senior informatics developer. Mr. Jacob Aaronson from OSU Biomedical Informatics IR&D team has successfully replaced Mr. Camerlengo’s role.
- Manuscript for the LDPMap algorithm developed in the collaboration between Dr. Huang and Dr. Xiang is accepted to a special issue in BMC Medical Genomics.

Conclusion

The project accelerated when the CRADA’s were executed. In the first two years, we optimized the primary genotyping and molecular methods, and the follow-on validation methods. We also expanded the capabilities of our highly-flexible DAPER database and software tools in the present reporting year. In the first year we invented an entirely novel approach to conducting genome wide genetic association (GWA) analysis – genomewide IUT analysis (GIA); and in the second year we further validated it. In this second reporting year, we integrated IUT and Bootstrapping as an additional innovation with outstanding utility. Dr. Alvarez’s presentation of these methods and results to leaders in the fields of genetics and canine genetics resulted in uniformly positive feedback from them (and multiple requests for collaboration). In addition, Dr. Huang has developed the LDPMap algorithm for enabling accurate query of biomedical terms in the database. We expect to publish the two revised manuscripts on GIA (one on methods, one on empirical cancer mapping) shortly, but the latter may be delayed while we analyze new supporting data acquired from Dr. Lindblad-Toh. In addition, we co-authored (Alvarez) a published study that was not based on the present military dog project, but which made use of the same data mining and analysis methods that will be used in our study. The LDPMap algorithm paper is accepted to BMC Medical Genomics. Dr. Rowell, one of our investigators (originally as a predoctoral student), moved on to conduct a postdoctoral fellowship with a pre-eminent dog geneticist at NIH and, after only a year there, is being recruited for a tenure track faculty position at OSU. Dr. Rybaczyk, another of our investigators (originally a postdoctoral fellow and promoted to research scientist) went on to be an NIH T32 Fellow at MSU, which is essentially a pre-faculty position. Dr. Alvarez was promoted to Associate Professor with tenure by OSU and is now under consideration for leadership training in the OSU College of Medicine.

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Appendices

I. Submitted National Institutes of Health grant application including Intersection Union Testing methodology (Aims 2, 3): Statistical techniques for optimized design and power in high-content genomics (Alvarez, PI; Huang, co-PI).

II. Accepted publication: Ren, K., A. Lai, et al. (2014). "Effectively Processing Medical Term Queries on the UMLS Metathesaurus by Layered Dynamic Programming." Accepted to BMC Medical Genomics.

Descriptive Title: Statistical techniques for optimized design and power in high-content genomics

Submission Title:

Opportunity ID: PAR-09-219

Opportunity Title: Exploratory Innovations in Biomedical Computational Science and Technology (R21)

Agency Name: National Institutes of Health

2. SPECIFIC AIMS. This application is in response to PAR-09-219, Exploratory Innovations in Biomedical Computational Science and Technology; it address research, development and application of analytical and statistical tools for interpretation of large biological data sets, and associated software. The flood of biological data has highlighted limitations to signal detection. Here we propose that combining optimized experimental design and novel uses of statistical methods can dramatically increase the power of signal detection. These approaches will be applicable to myriad data types and their integration. However, this proposal will demonstrate validity using a highly innovative approach to complex genetics. We will conduct a Genome Wide Association (GWA) study using high density genotyping that not only provides binary single nucleotide polymorphism (SNP) allele data, but also total SNP signal and allele ratios (which can be affected by DNA copy number variation, CNV). In Preliminary Studies we demonstrate the feasibility of using allele ratios as continuous variables to map disease loci. This is the first such GWA study of comprehensive CNV information without prior classification of markers as CNV. Our ***hypothesis*** is that implementation of our algorithm on multiple (experimentally standardized) groups dramatically increases the power to detect biological signal.

Experimental design. The now common use of thousands or tens of thousands of subjects in genetic studies can be attributed to genetic heterogeneity/complexity and diverse confounds of meta-analysis. A major limitation is the extreme multiple-testing burden in GWA, which is commonly done by Chi-Square testing of one million markers. In Preliminary Studies, we address these issues by 1) conducting complex disease mapping studies in one dog breed, which has 100-fold reduced genetic variation compared to humans, and 2) using multiple, but experimentally identical, case-control sets or batches. In this way, there are reduced numbers of disease-associated markers in a simpler background and we can apply an Intersection Union Test (IUT) across experiments (in place of Bonferroni multiple-test correction). ***Computational statistics.*** The overarching goal of the proposed analytical approaches is based on the information theory concept that the more manipulations or corrections are implemented, the more information is lost. We propose here that this loss of information can be eliminated in diverse types of biological data by integrating two elements. In the first, we use analysis of covariance (ANCOVA) to correct continuous variable data for latent known biological confounders such as group membership. In the second, we make use of optimized study design (specifically, using multiple case-control groups for a given experiment) to perform IUT. Others recently validated a similar use of IUT independently. In Preliminary Studies, we demonstrate validation of the integrated ANCOVA and IUT. We confirm that the use of IUT on multiple sets is a more effective solution to the three reversal paradoxes (Yule-Simpson, Lord's, and suppression) which share the characteristic that the association between two variables can be reversed, diminished, or enhanced when another variable is statistically controlled for. Notably, we are first to address these in the context of continuous genomic variables.

Aim 1: ***Demonstrate on large datasets the ability of ANCOVA to correctly identify biologically relevant phenomena that are linked to a disease trait.*** ANCOVA has been applied to correct for baseline variables in various fields, such as psychology and epidemiology. Despite similarities in variable types, data structure, and confounds, ANCOVA has never been applied to large scale genetic datasets. We will analyze different types of genomic datasets (our own and from the public domain) with well-established population confounds and show that ANCOVA is the most effective way of removing those.

Aim 2: ***Application of IUT for genetic analysis, allowing for multiple corrections without manipulation of individual datasets.*** We propose to demonstrate the ability of IUT to detect complex genetics in a disease phenotype and how combining IUT with ANCOVA will allow the detection of genetic determinants. The non-obvious advancement of this method is that it incorporates information theory by minimally altering the data before analyzing it. This retains the maximum amount of information for each measure. It also does not assume linear relationships with latent variables.

Aim 3: ***We will validate our claim that ANCOVA and IUT are more powerful than traditional techniques.*** We will replicate a published canine complex-genetics mapping study using fewer individuals to demonstrate that our technique is able to detect the same loci in addition other variants missed by traditional techniques. We will also conduct a novel GWA study of a human medically relevant complex trait in a second dog breed.

3. RESEARCH STRATEGY

(a) SIGNIFICANCE

We will develop and implement analytical and statistical tools (and software) for interpretation of large biological data sets. The explosion of biological data has made prominent several limitations to signal detection.[1] We demonstrate in Preliminary Studies that combining optimized experimental design and novel application of statistical approaches can dramatically improve signal detection. These methodologies will be applicable to analytical challenges of myriad data types and their integration [2], including genomics [3], high throughput (HT) sequencing [4], population biology and genetics [5,6], and gene/organism/environment interactions [7]. The improvements described here address the basic concept of information theory that more manipulations of data equals more information loss. Among the areas addressed, are 1) application of analysis of covariance (ANCOVA; [8]) to correct continuous variable data for latent known biological confounders as well as potentially avoiding the three reversal paradoxes (Yule-Simpson, Lord's, and suppression), which share the characteristic that the association between two variables can be reversed, diminished, or enhanced when another variable is statistically controlled for [9,10], and 2) multiple new applications of the Intersection Union Test (IUT; [11]), including GWA, as was independently developed by another investigator very recently [12]. This proposal thus offers solutions and software to address critical barriers to genomic analysis, simultaneously improving scientific knowledge and technical/analytical capabilities.

(b) INNOVATION

Multiple phenotypic traits (such as height or weight) are often treated as independent from the effect under study, but that neglects the reality that many traits are linked to other genetic and environmental modifiers. Others incorporate and calculate variances based on environmental or geographic stratifications. However, this ignores synergism between the organism, its immediate surroundings, and the greater environment. While it is not possible to measure and analyze every part of the environment, some baseline state must be identified from which deviation can be measured to test *a priori* hypotheses. In the absence of this uniform baseline, almost all statistical measures will fail to adequately detect regions of interest. ***This application will demonstrate feasibility and innovation in preliminary studies (c.5) using an entirely new approach (ANCOVA/IUT) to conducting genome wide association (GWA) genetics based on continuous variable data.*** An important challenge to GWA that relates to these issues above is population structure (i.e., correcting genetic studies for non-disease-associated allele frequencies that vary in human populations). Two common ways to address this are traditional meta-analytic techniques and IUT. But these approaches are selected more out of necessity than experimental design concerns. The majority of combinatorial studies have focused on publicly available datasets. Each of the individual datasets contains differing degrees of artifactual bias and other, potentially unrelated, variables. Oncomine's [13] and other algorithms applying this strategy to gene-expression have some success but it has not been the panacea originally prognosticated.[14]

Multivariate and integrative analyses can potentially solve many issues associated with genome wide studies.[15,16] However, they are limited by their ability to synthesize data into useful parcels of information that are applicable clinically or to research. Integrative analysis has the benefit of alternative testing. While multiple testing using the same measures and techniques increases error rates [17], alternative testing allows measurement of the same effect using different types of measures. As these are subjected to different analytic techniques, the posterior probability of false positives is reduced. Even with this strength, it is limited by biases and assumptions associated with individual measures. Ultimately the question of how to appropriately identify genetic contributions independent of latent confounds has not been conclusively answered. The gold standard for analyses is univariate testing. While geneticists talk about penetrance in relation to populations and percentages, the statistical actuality is that penetrance describes odds ratios. Establishing causation and deviation from population norms using case-control, linkage, or association analyses requires certain assumptions to be accepted that biologically may or may not be perilous to the analysis. While this is important to ethologists and population geneticists, attempting to compensate/account for these phenomena hinders and complicates analyses. We are interested in identifying biological outcomes that are well described and were

not concerned with tangential characteristics of the effect. To this end, we sought to isolate rather than compensate for effects. When examining multidimensional data it is easy to disregard the interaction of dimensions. Most dimensional reduction techniques measure and condense data so that interdimensional effects can be quantified. Priming effects can drastically alter these techniques and limit their usefulness. For this reason we applied ANCOVA [8] to remove independent effects from dependent effects prior to dimensional reduction. Here we show adjusted and un-adjusted measures to illustrate how the application of ANCOVA prior to traditional techniques is capable of increasing the sensitivity of a study, as well as the potential to correct for the reversal paradoxes (c.5. P.S., Study Design) by comparison to traditional normalization techniques.

(c) APPROACH

c.1. Research team. The multidisciplinary team is ideally suited for this project. Dr. Alvarez (PI) is PI in Molecular and Human Genetics, Nationwide Children's Hospital Research Institute, with a tenure track academic appointment at The Ohio State University College of Medicine. He has extensive expertise in molecular and human genetics and genomics, bioinformatics, and, from management level industry experience (Novartis Research), the discovery and validation of new drug targets and biomarkers. Dr. Leszek Rybaczyk (Research Scientist, Alvarez Lab) is expert in statistical bioinformatics. Dr. Huang Kun (Co-I) is co-director of the OSU-CCC Biomedical Informatics Shared Resource. His research is focused on developing bioinformatics tools for systems biology and research. Here he will be responsible for developing and implementing the software package. The advanced statistics expertise will come from a long term collaborator of the three investigators named above, Dr. Pramod K. Pathak (consultant, MSU). He is a theoretical and applied statistician with specific interests in statistical methods and their applications to biomedical research, sampling and resampling methods, computational statistics, reliability, and optimization problems in statistics.

c.2. Research strategy (RS). ***Note:*** As the approach has statistical components addressing different biology, we will explain the approach once, in Research Strategy, and establish feasibility in Preliminary Studies.

RS Aim 1. We propose to address these gaps by applying statistically proven methodologies in novel ways. ANCOVA has been applied in various fields such as psychology [18] and epidemiology [19] to correct for baseline variables.[20] Despite the similarities in variable types, data structure, and problems with confounds [19] ANCOVA has never been applied to large scale genetic datasets. **Aim 1:** Demonstrate on a large dataset the ability of ANCOVA to correctly identify biologically relevant phenomena that are linked to a disease trait. The rationale and technical approach for this aim are well elaborated in c.5. Preliminary Studies. Canine genetic data similar to those generated in Preliminary studies will be generated from 1) 36 Scottish Deerhounds: 18 osteosarcoma cases and 18 controls (i.e., three case-control batches of six and six), as well as 2) 36 Doberman (18 with cervical spondylomyelopathy and 18 controls (i.e., three case-control batches of six and six). In addition, we will analyze diverse genomic datasets from the public domain (including human SNP GWA, gene expression, and HT-sequencing). For example, by using TCGA data, in which the same patient's tissue was assayed on different microarrays in different laboratories, using an ANCOVA approach we will identify the most biologically relevant factors. We will expand that by looking not only at the cancer type, but also at the laboratory where the tissue was processed; the date on which it was processed, etc., and identify/potentially remove such intrinsic errors.[21] ***Power analysis.*** Based on our ongoing genetic studies (see Preliminary Studies), we assumed that potentially relevant SNPs will reduce the total of 173,000 SNPs to 1700 [MD Anderson Bioinformatics server with power of 0.8, acceptable false positives of 1, SD of 0.7. With the sample size of 36 dogs in each breed (18 cases and 18 controls) we will have 80 % to detect 2-fold differences in B allele frequency between cases and controls for candidate SNPs of interest (per SNP alpha = 0.00059). This is conservative, as ANCOVA and IUT would only reduce the variance.

RS Aim 1 Potential pitfalls and contingencies. (1) A limitation to using the integrated ANCOVA/IUT on biological data is that it is only applicable for continuous variable data. While this excludes, say, conventional binary-genotype GWA analysis, we address this need with the development of an IUT-alone approach; this use is now validated by us (see c.2. RS Aim 3 Expected results, Example 1) and by a second independent group.[12] Moreover, much genetic data (e.g., array CGH, HT-sequencing) and most genomic data has continuous variables (microarray and HT-sequencing based RNA expression and epigenetics, proteomics, metabolomic, etc.). (2) Another potential concern is the need for clear understanding of appropriate data structure. For that reason, we chose to make this proposal not only about the statistical methods, but also

about experimental design. We will make a major effort to document the proper use of these algorithms in publications and software Help documentation. (3) Lastly, these methods are computationally intensive. This will not affect us, as Dr. Huang (Co-I) is Director of Bioinformatics and has access to the OSU Supercomputer Center. Despite the computational demands, the methods proposed here offer analytical abilities that are unique and state of the art, and are sure to gain wide use. We believe that our optimization studies and careful statistical/software instructions will facilitate the most efficient implementation of our algorithms.

RS Aim 2. A second statistical technique, the Intersection Union Test, has been gaining use in the genomics field.[22] The IUT increases power, but also increases type I error as the number of comparisons increases.[12] However, because of the many latent confounds that cannot be accounted for in most genomic work, the IUT is the most elegant solution to reducing these errors.[23] For instance, in large datasets where a multitude of tests are conducted under traditional techniques, a multi-testing correction would need to be applied. However, as we previously demonstrated using the IUT, the probability of any specific false positive decreases exponentially with the addition of new datasets.[24] This is because the probability of detecting the same false positive in two independent datasets is the multiple of α, traditionally 0.05. For two datasets the probability of the same false positive being detected is 0.0025, for three it is 0.000125, and so on. This can compensate for even large datasets. In datasets with 173,000 variables (SNP arrays used in preliminary studies), using between 4 and 6 independent datasets would eliminate all false positives. Conversely if the same signal is being detected in 6 datasets the probability that it is due to chance is of the order $1.5x10^{-8}$. **Aim 2:** IUT is powerful new tool for genetic analysis and allows for multiple corrections without manipulation of individual datasets. We purpose to demonstrate the ability of IUT to detect complex genetics in a disease phenotype and how combing IUT with ANCOVA will allow the detection of genetic determinants and potentially explain penetrance. The non-obvious advancement of this method is that it incorporates information theory by minimally altering the data before analyzing it. This retains the maximum amount of information for each measure. The IUT is also not hampered by many of the assumptions of other tests.[20]

RS Aim 2 Potential pitfalls and contingencies. The IUT is dependent on having a common variable across all data sets used in the analysis. This variable can be very broad such as dog breed or very narrow such as a molecular phenotype. Regardless, the IUT will only answer questions related to the common variable among data sets. One way to correct for that is in the initial study design. The study design should take into account all of the limitations associated with the various statistical tests a priori. As we recently discussed in a publication, applying the IUT to unrelated data sets will result in the elimination of all signal.[24]

RS Aim 3 rationale. Large scale studies that use traditional GWA require large patient populations to achieve adequate power (and have yet to explain a significant portion of the heritability associated with most diseases).[25,26] This has serious pragmatic and ethical implications.[27] It also poses several experimental design problems as independent irrelevant variables – e.g., in genetics, population structure, can overpower the effect of interest.[28] Manipulation of data by Principal Component Analysis (PCA) after merging, or applying normalizations, hinge on the assumption that the interactions are linear. If the interactions are non-linear, applying these corrections can make analysis more difficult.[29] **Aim 3:** We propose to demonstrate that ANCOVA and IUT are more powerful than the traditional techniques by identifying a study and replicating that study using fewer patients and demonstrating that our technique is able to detect the same signal in addition other variants missed by the more traditional techniques.

RS Aim 3 Genetic studies experimental plan. As we did in Preliminary Studies (c.5., using the same Illumina 173,000 SNP array), we will conduct GWA analysis of two complex traits, each with high incidence in a dog breed. ***Mapping (1)*** As validation of a complex trait that has been mapped using a conventional genetic approach and published, we will map osteosarcoma in Scottish Deerhounds (one locus of dominant effect with evidence of linkage (Z_{max}=5.766)).[30] The original work used a 4-generation pedigree where 60 Deerhounds were genotyped and the genotypes of 70 others were inferred, for a total of 130 dogs. We will replicate that study using the methods developed in this proposal to conduct GWA (ANCOVA/IUT on B allele frequency data and IUT on allele/genotype data) on 18 Deerhound cases and 18 controls (i.e., three case-control batches of six and six). ***Mapping (2)*** In order to immediately draw high impact attention to our innovative approaches, we

propose to conduct GWA of a prominent breed-specific complex-genetic condition with high human relevance – "wobblers" or cervical spondylomyelopathy in Doberman Pinschers (reported to explain 2.5% of proportional mortality in the breed).[31,32] We have been collaborating for over a year with Ronaldo da Costa, our OSU colleague who is a leading authority in this.[32] We are currently conducting pedigree analysis on ~1000 Dobermans (showing strong evidence of heritability; data not shown), and have initiated collection of blood/DNA samples. Using the Doberman wobblers pedigree, we will select optimal informative dogs to conduct a mapping study with 18 cases and 18 controls (i.e., three case-control batches of six and six). ***Power analysis.*** See c.2. RS Aim 1, end of first paragraph.***Follow up to broad mapping:*** depending on the type/strength of the evidence and the length of the haplotypes, we will conduct either fine mapping in related breeds that share a similar phenotype, sequence implicated haplotypes using sequence capture, or characterize transposition events, structural variation or DNA methylation status (see PI (Alvarez) biosketch, which demonstrates successful funding of grants in this area from NIH, DoD CDMRP and AKC-CHF). The PI is expert in genomics and sequence and evolutionary biology analyses that will be required to fully evaluate genetic variants and their possible disease effects.[32-38]

RS Aim 3 Expected results. We predict that in *Mapping (1)* we will identify the same locus published previously (leading to refining the locus through recombination in both breeds), and that we will identify other loci associated with osteosarcoma risk – both SNP alleles and B allele frequency changes suggestive of CNV or of effects resulting in allele-specific SNP genotyping bias from amplification step [[39]]. As Deerhounds are relatively closely related to Greyhounds, we also expect to find some loci shared between the two, which would provide convincing replication of the findings in our preliminary studies. We predict that in *Mapping (2)* we will find wobblers-associated variants. For both mapping studies we expect to identify loci that could not have been found using conventional genetic analyses. *Example 1*, in preliminary GWA studies applying IUT to binary genotype calling of the same Illumina SNP array data used in c.5. Preliminary Studies, we identified a genome wide significant locus that would not have been identified by conventional Chi-Square GWA analysis (not shown). Strikingly, two of the three case-control groups had increased frequency of the SNP allele associated with high risk, but the third group had reduced frequency of the same allele associated with reduced risk. We propose that, due to reversal paradox effects [[9,10]], many such findings cannot be detected by conventional GWA. We also expect to identify candidate genes (e.g., some osteosarcoma candidate haplotypes have no more than one gene) and variants (e.g., through sequence capture) within association loci. *Example 2,* in Preliminary Studies we demonstrate the use of ANCOVA/IUT to identify continuous variable differences in B allele frequencies associated with osteosarcoma risk. This would not be possible with current approaches that map binary SNP alleles (and cannot be detected indirectly by tag-SNPs in LD when the variants are relatively recent). Such variation may be indicative of genetic effects never before sampled genome wide for GWA, such as CNV or isothermal amplification bias [[39]] in Illumina Infinium SNP genotyping (e.g., due to DNA methylation, structural variation, and retrotransposition events). If our expected results materialize, as is strongly supported by our preliminary studies, they would establish the superior power and preservation of information in the innovative experimental design and analyses we propose; and it would open the door to studying the most common (and with highest mutation rates) types of genetic variation [[38]] for the first time.

RS Aim 3 Potential pitfalls and contingencies. Our preliminary studies support the feasibility of applying very well-established statistical methods for novel biological data analyses. For example, applying an IUT approach to GWA using binary genotype data, identified a SNP locus at genome wide significance; but no locus reached significance using conventional Chi-Square analysis on the same genotype data (see Example 1 in previous section). Notably, others have recently independently validated that same application of IUT.[12] A second example is the fact that the ANCOVA/IUT mapping approach identified several loci that were covered by multiple significant SNPs, including five SNPs in a 600,000 kb region of chr6; the odds of the observed physical genome distribution being a random effect are infinitesimally low. The greatest challenges in the field of GWA are validation of association and identification of causative mutations. These remain potential pitfalls for us, but we are encouraged by the fact that our osteosarcoma GWA (using IUT of conventional binary genotypes) in Greyhounds identified one (of 19 significant) SNPs within the 4.5 Mb interval identified for

linkage to osteosarcoma in the closely related Scottish Deerhound. This ability to fine map across related breeds is one of the major strengths of dogs, as are the reduced phenotypic and genetic heterogeneity.[40] For the mutation detection, we will be challenged as is everyone, but 1) we have improved chances over most others because we will have more loci to prioritize for specific molecular approaches based on our types of findings (say, structural variation vs. DNA methylation), and 2) we have the technical and computational expertise, and are using the most cutting edge methodologies.

c.4. Software development

All the algorithms developed in this project will be integrated into an open source R package using R and Bioconductor functions and packages. The package will be tested on both stand-alone workstation and also parallel computing environment including two clusters available at OSU (one in the Ohio Supercomputer Center, one in the Dept. of Biomedical Informatics). The packages will be released on a project website and freely available to public. In addition, we will submit it to Bioconductor in compliance with the testing and inclusion criteria. If time permits, we will also consider integrating the R package into a web tool using web interface tools such as the *Rcgi* package (a CGI WWW interface R).

c.5. Preliminary studies & Demonstration of proposed experimental approach

Note: To demonstrate the novelty and significance, and the experimental plan for all three Aims, we devote significant space in this proposal to describe our preliminary studies (two manuscripts in preparation)..

Study design (ANCOVA/IUT approach), canine osteosarcoma (OSA). Dog breeds have ~100-fold less genetic variation than humans. Greyhounds were split over one hundred years ago into racing and show sub-breeds (registered NGA and AKC, respectively). Strikingly, racers have the highest OSA rate (25% incidence) of any breed, whereas show dogs have no increased risk.[41,42] We thus designed a study of a complex genetic trait in an outbred mammal, but used one of the simplest such contexts possible. Genotyping of these dogs was performed using the highest density SNP array available in dogs (Illumina HD, 173,000 feature; fewer SNPs than humans due to the highly extended linkage disequilibrium (LD) in dogs). Importantly, this genotyping platform provides not only the presence or absence of the binary A or B alleles at each marker, but also the signal intensity of the marker and the ratio of the two alleles (referred to as B allele frequency, BAF). We conducted the SNP genotyping in three OSA positive-negative (case-control) groups in order to 1) using ANCOVA to adjust for group membership as well as potentially addressing the three reversal paradoxes (Yule-Simpson, Lord's, and suppression), which share the characteristic that the association between two variables can be reversed, diminished, or enhanced when another variable is statistically controlled for [[9,10]]; and 2) enable the use of IUT in place of GWA by Chi-Square analysis with Bonferroni multiple testing correction. Specifically, we genotyped batches of 12 dogs in the combination of 4 OSA racers, 4 OSA free racers (OFR) and 4 show (AKC).**Statistics & Results:** Data was analyzed using Illumina GS and Partek GS. Sample attributes (incl.

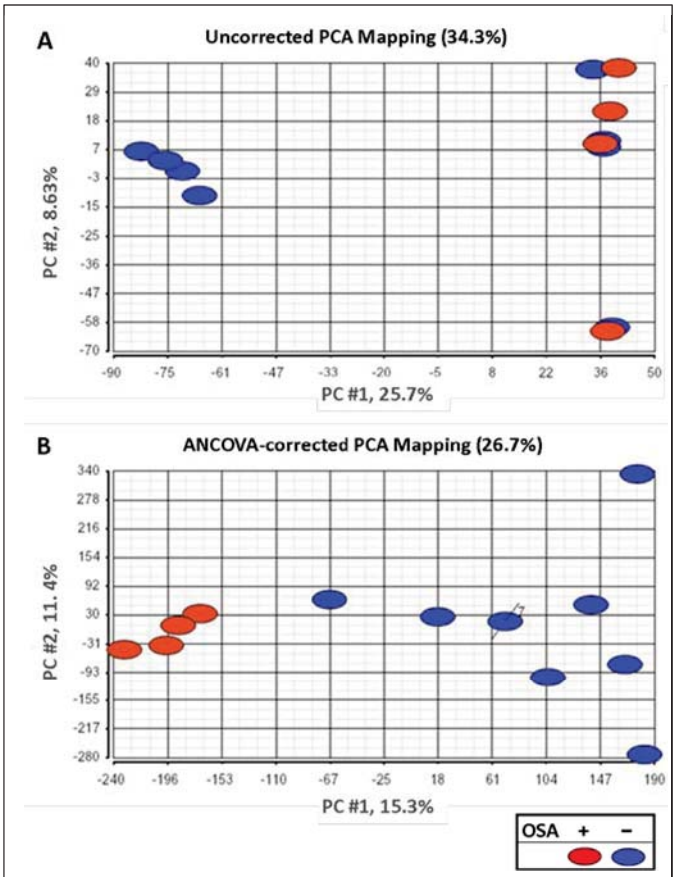

Fig 1. Application of ANCOVA. Correction of Greyhound osteosarcoma (OSA) positive and negative continuous variable genotypes (B allele frequencies). (A) Uncorrected analysis shows population structure effects: separating OSA positive and negative racers apart from negative AKC show Greyhounds. (B) ANCOVA-corrected analysis cleanly separates OSA positive and negative dogs.

racing/show and disease status) were used to assign animals to conditions for ANCOVA corrections. ANCOVA is based on regressions and when used as a statistical test assumes that covariates are independent variables. In our ANCOVA procedure we used it to establish weighted averages so that groups that are biologically similar have the same regression slope. Linear models in biological contexts have been heavily criticized. In this procedure a linear model is entirely appropriate since we are classifying based on known biological traits. Although this does render the measures arbitrary it allows for effects to be isolated that can be subjected post hoc to other tests. Figure 1 demonstrates the effects of ANCOVA isolation on principal components associated with the phenotype of interest. Before correction, two low risk groups (AKC and OFR) fail to cluster according to risk due to population structure. Regression lines were computed for the appropriate factors and interaction values were transformed and weighted to correct for the slope of the generalized linear model. We next calculated the covariance matrix of the loading values for each dataset and conducted IUT using a threshold of ±0.6. Many publications have reported that Pearson correlation (r) values of 0.4 are biologically significant. Here we used 0.6 assuming it most likely captures the most informative SNPs.

Table 1. Analysis of informative SNPs using ANOVA for multiple categories of risk.

SNP	Chr	Position
BICF2S23318678	3	22278940
BICF2P756511	3	34630563
BICF2S22958963	3	34806577
BICF2S23713946	5	3741194
G320f26S259	5	3814438
BICF2P959468	5	24064707
BICF2S23647041	5	25563084
BICF2S23746914	6	71831263
BICF2S22933176	6	72089371
BICF2P643804	6	72282176
BICF2P878053	6	72314083
BICF2S23332924	6	72453644
G439f54S214	7	23851944
TIGRP2P97627	7	49152204
BICF2P989771	9	27058611
BICF2P395540	12	67862864
BICF2P998637	14	39888317
BICF2S23147465	14	51418412
BICF2S2339350	18	23106621
BICF2S23348607	18	23130080
BICF2P950849	18	37553821
TIGRP2P335678	25	54551661
BICF2P691768	28	42235397
BICF2P681391	31	39698895
BICF2P623089	34	30054450

A list of potential candidate SNPs from the ANCOVA/IUT was identified and used to filter genotype information. Genotypes were subjected to a Chi-Square test of association for osteosarcoma risk. Non-significant genotypes were eliminated from the analysis. Once only SNPs that are loaded with the most meaningful measures remained we conducted t-tests to determine if they were capable of discriminating between the two training populations. This procedure revealed that the osteosarcoma free racers and the AKC show greyhounds which have below average incidence rate clustered together and the first principle component explained the osteosarcoma risk variability initially masked by the effects of the population difference (Fig. 1B). We then went on to determine whether it was a genotypic effect such as haplotypes or if some other mechanism was associated with the differential risk in these two populations. Intriguingly, regions associated with altered risk could not be identified based on haplotypes alone. However, the signal was derived from alterations in B allele frequency that correctly categorizing dogs across unrelated datasets. The genome wide significant hits are shown in Table 1. Encouragingly, several regions are detected by multiple SNPs (colored), including five SNPs in a 600,000 kb region of chromosome 6.

Preliminary studies conclusions. Here we presented the first GWA study of osteosarcoma in any organism, and reported approximately twenty hits. Our approach showed how population structure can affect the ability to detect biologically relevant genetic effects. In addition, this is the first work to detect genome wide significant association signal using continuous variable genotype data (B allele ratios) and ANCOVA/IUT; we propose those loci are a combination of CNVs and genetic/epigenetic variants with differing amplification bias [[39]] in the SNP genotyping protocol. This is consistent with Dr. Nadeau's suggestion that the missing heritability may lie in unexplored genome regions or "in largely untested classes of genetic variation."[43] Beyond the analysis shown here, we conducted a second GWA analysis of the same data, but applying only IUT using binary allele calls – see c.2., RS Aim 3, Expected results and Potential pitfalls and contingencies. That analysis suggested validation of the study, as one of 19 genome wide significant hits is within the 4.5 Mb interval linked to osteosarcoma in Deerhounds. Moreover, we identified SNPs that could not be identified by conventional approaches due to the reversal paradoxes.

Application summary: We propose to develop novel applications of validated statistical approaches to enable greatly improved analysis of continuous-variable biological data. This and the new applications of IUT will be widely used for genomic and integrative analyses.

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Effectively processing medical term queries on the UMLS Metathesaurus by layered dynamic programming

Kaiyu Ren[1,2], Albert M. Lai[1], Aveek Mukhopadhyay[2], Raghu Machiraju[2], Kun Huang[1], Yang Xiang[1§]

[1] Department of Biomedical Informatics, the Ohio State University, Columbus, OH 43210, USA

[2] Department of Computer Science and Engineer, the Ohio State University, Columbus, OH 43210, USA

Contact: Yang Xiang

[§]Corresponding author

Email addresses:

YX: yxiang@bmi.osu.edu

Abstract

Background

Mapping medical terms to standardized UMLS concepts is a basic step for leveraging biomedical texts in data management and analysis. However, available methods and tools have major limitations in handling queries over the UMLS Metathesaurus that contain inaccurate query terms, which frequently appear in real world applications.

Methods

To provide a practical solution for this task, we propose a layered dynamic programming mapping (LDPMap) approach, which can efficiently handle these queries. LDPMap uses indexing and two layers of dynamic programming techniques to efficiently map a biomedical term to a UMLS concept.

Results

Our empirical study shows that LDPMap achieves much faster query speeds than LCS. In comparison to the UMLS Metathesaurus Browser and MetaMap, LDPMap is much more effective in querying the UMLS Metathesaurus for inaccurately spelled medical terms, long medical terms, and medical terms with special characters.

Conclusions

These results demonstrate that LDPMap is an efficient and effective method for mapping medical terms to the UMLS Metathesaurus.

Background

Efficiently processing and managing biomedical text data is one of the major tasks in many medical informatics applications. Biomedical text analysis tools, such as MetaMap [1] and cTAKES [2], have been developed to extract and analyze medical terms from biomedical text. However, medical terms often have multiple names, which make the analysis difficult. As an effort to standardize medical terms, the

Unified Medical Language Systems (UMLS) [3] maintains a very valuable resource of controlled vocabularies. It contains over 200 million medical terms (also known as "medical concepts"). Each medical term is identified by a unique id known as a Concept Unique Identifier (CUI). The UMLS also records relations between medical terms. As a result, mapping biomedical text data to the UMLS and mining UMLS associated datasets often yield rich knowledge for many biomedical applications [4] [5] [6] [7] [8].

In order to effectively query or use the UMLS, one of the fundamental tasks is to correctly map a biomedical term to a UMLS concept. Currently, there are a number of publicly available tools to achieve this goal. One notable approach is to use the official UMLS UTS service (UMLS Metathesaurus Browser) available on the UMLS official website (https://uts.nlm.nih.gov). Users are able to input a medical term and the system will return a query result. MetaMap [1], which has been developed and maintained by US National Library of Medicine, has become a standard tool in mapping biomedical text to the UMLS Metathesaurus. cTAKES [2] is an open-source natural language processing system that can process clinical notes and identify named entities from various dictionaries, including the UMLS.

However, after having been using these tools in our research, we found that they do not work well in mapping medical terms that are just slightly different from the terms in the UMLS. For example, the UMLS Metathesaurus Browser, MetaMap, and cTAKES fail to process the query term "1-undecene-1-O-beta 2',3',4',6'-tetraacetyl glucopyranoside" even if it has only one character different (missing "-" between "beta" and "2") from the official UMLS concept "1-undecene-1-O-beta-2',3',4',6'-tetraacetyl glucopyranoside". This drawback makes it hard to handle many real world data such as Electronic Health Records, which contain a lot of noisy information

including missing and incorrect data [9]. In addition, they often fail to handle long medical terms even if those terms are identical to the terms in the UMLS. For example, the Metathesaurus Browser cannot handle query terms with more than 75 characters, and sometimes cannot even accurately answer a query term that exactly matches a concept name in the UMLS (see discussions in the result section). MetaMap and cTAKES, on the other hand, often breaks down a long medical term into several shorter terms. For example, if we query MetaMap with a clinical drug "POMEGRANATE FRUIT EXTRACT 150 MG Oral Capsule", we get several UMLS concepts such as "C1509685 POMEGRANATE FRUIT EXTRACT", "C2346927 Mg++", and "C0442027 Oral", instead of this drug concept which has a unique CUI C3267394 in the UMLS. The situation becomes even worse when medical terms contain special characters, i.e., characters other than numbers or letters, such as "{", "}", " (",")","-", etc. For example, MetaMap completely fails to find any relevant CUI to the medical concept "cyclo(Glu(OBz)-Sar-Gly-(N-cyclohexyl)Gly)2". These drawbacks are very undesirable when handling biomedical texts. By studying the UMLS Metathesaurus, we found that a significant number of medical terms are quite long. About 10.7% of UMLS concepts contain at least 75 characters (including white spaces), and about 50.9% of UMLS concepts contains at least 32 characters. In addition, a large amount of medical terms contain special characters. More than 61.3% of UMLS concepts contain at least one special characters and about 11% of UMLS concepts contains at least 5 special characters. In fact, we found many special characters are optional in a medical term. For example, term “Cyclic AMP-Responsive DNA-Binding Protein” and term “Cyclic AMP Responsive DNA Binding Protein” both refer to the same concept "C0056695" in the UMLS Metathesaurus, though the latter is missing two "-". The UMLS handles a medical term with different

names by including multiple common names in the Metathesaurus. Given the fact that in many cases special characters are optional, it is practically impossible to let Metathesaurus contain all possible names. Considering a UMLS concept with 20 special characters, if each special character may be replaced by a white space, then there are approximately 1 million aliases for this concept alone, not to mention that more than 0.3% of UMLS concepts contain 20 special characters or more.

This problem is in fact related to the classical spelling correction problem in which a misspelled word will be corrected to the most closely matched word. The classic measurement of dissimilarity between two words based on several distance functions, such as edit distance [10], hamming distance [11], and longest common subsequence distance [12] [13]. Thus the spelling correction is essentially finding a valid word with the minimum distance to the misspelled word. Quite a few dynamic programming algorithms have been proposed to solve this problem. Readers can find a survey of these algorithms in [14]. In recent years, spelling correction has evolved to perform query corrections. This correction is often a task of context sensitive spelling correction (CSSC), where corrections will be geared towards more meaningful or frequently searched words [15]. Thus, it is a good idea to use the query log to assist the correction [16].

Unlike many query applications, it is not sufficient to return a frequently searched medical term that best matches the query based on search history, not to mention that such history data is often not available. Accurately identifying a specific biomedical term, such as a drug name or a chemical compound, is demanded by many biomedical applications. Given this consideration, classical spelling correction techniques are more preferable than the CSSC for matching biomedical terms to UMLS concepts. However, we found that the classical dynamic programming algorithm is too slow for

this task because of the huge volume of terms in the UMLS Metathesaurus. In addition, it is unable to effectively handle a term with missing words (e.g., "gastro reflux" has a large distance to "gastro oesophageal reflux" though the two terms usually means the same thing), or words not in their usual order (e.g., "lymphocytic leukemia chronic" has a large distance to "leukemia chronic lymphocytic").

The background described above motivated us to find an efficient and accurate medical term mapping method for the UMLS. To tackle this challenge, in this work we propose a Layered Dynamic Programming Mapping (LDPMap) approach to query the UMLS Metathesaurus.

Methods

We use Longest Common Subsequence (LCS) to measure the similarity between two words. Given two words *A* and *B*, their similarity is defined as:

$$WordSimilarity(A, B) = 2*|LCS(A,B)| / (|A|+|B|);$$

This similarity measure is a variation of the longest common subsequence distance [12]. We can observe that $WordSimilarity(A, B)$ ranges between 0 and 1. In addition, $WordSimilarity(A, B) = 1$ if and only if *A* and *B* are identical, and $WordSimilarity(A, B) = 0$ if and only if *A* and *B* shares no common letters.

The function $WordSimilarity(A, B)$ is the basic building block for LDPMap. In the UMLS, each concept is a sequence of words. We define the similarity between two concepts $\alpha_n = (A_1, A_2, \ldots, A_n)$ and $\beta_m = (B_1, B_2, \ldots, B_m)$ as:

$$ConceptSimilarity(\alpha_n, \beta_m) = max\left(\sum_{(i,j)\in R} WordSimilarity(A_i, B_j)\right);$$

Similar to word similarity, in our query we will normalize the concept similarity by the number of words contained in each concept. We can observe that normalized

concept similarity score ranges between 0 and 1. If two concepts are identical then this score is 1.

$$NormConceptSimilarity(\alpha_n, \beta_m) = 2*ConceptSimilarity(\alpha_n, \beta_m)/((|\alpha_n| + |\beta_m|);$$

The key issue in the above definition is *R*, which is a matching relation between words in α and β. We have two constraints on *R*, which leads to two different foci.

Constraint 1: There do not exists two matching pairs (*i*,*j*), (*x*,*y*) in *R* such that $i=x$ or $j=y$.

Constraint 2: In addition to constraint 1, for any two matching pairs (*i*,*j*), (*x*,*y*) in *R*, either $i<x$ && $j<y$, or $x<i$ && $y<j$.

Constraint 1 converts the concept similarity problem into a maximum weighted bipartite matching problem [17]. Considering a bipartite graph built on two vertex sets α_n and β_m with word similarities being the edge weights, finding a highest score for concept similarity under Constraint 1 is equivalent to find a maximum weighted matching for the bipartite graph. This model is particularly helpful for identifying the similarity between two terms regardless of their word ordering. We used this as one of the measurements in our final query workflow (Figure 1) and implemented this by maximal weighted matching.

In the following section, we will focus on concept similarity calculation under constraint 2, which regulates that the similarity comparison between two terms shall follow the word orders in those terms, similar to the LCS problem in which matching between two words shall follow the character orders. Thus, the concept similarity calculation problem can be considered as a macro level similarity calculation where each unit is a word instead of a letter as in the case of word similarity calculation. This model has a lot of advantages as we will see in the following section.

Suboptimal Structure of the Concept Similarity under Constraint 2

Our next question is how to perform the concept similarity calculation. Unlike word similarity calculation in which each match outcome is a binary result (i.e., the same letter or a different letter), each match in the concept similarity calculation is a word similarity value between 0 and 1. The algorithm for the word similarity calculation cannot be applied to the concept similarity calculation. However, we find the concept similarity calculation also has a suboptimal structure as follows:

if i=0 or j=0

$ConceptSimilarity(\alpha_i, \beta_j) = 0$

else

$ConceptSimilarity(\alpha_i, \beta_j) = max(ConceptSimilarity(\alpha_{i-1}, \beta_{j-1}) + WordSimilarity(A_i, B_j), ConceptSimilarity(\alpha_i, \beta_{j-1}), ConceptSimilarity(\alpha_{i-1}, \beta_j))$;

The above suboptimal structure is true because for any two words $A_i \in \alpha_i$, $B_j \in \beta_j$, there are at most three possible cases:

(1) $(i, j) \in R$, i.e, Both A_i and B_j are used in the matching. Then $ConceptSimilarity(\alpha_i, \beta_j) = ConceptSimilarity(\alpha_{i-1}, \beta_{j-1}) + WordSimilarity(A_i, B_j)$;

(2) B_j is not used in the matching, then $ConceptSimilarity(\alpha_i, \beta_j) = ConceptSimilarity(\alpha_i, \beta_{j-1})$;

(3) A_i is not used in the matching, then $ConceptSimilarity(\alpha_i, \beta_j) = ConceptSimilarity(\alpha_{i-1}, \beta_j)$.

Note that we do not consider it a valid case that neither A_i nor B_j is used in the matching. In this case, we can always choose to make them matching without violating Constraint 1 and result in a higher or at least equal concept similarity score.

Main Algorithms

Given the suboptimal substructure, we can design a dynamic programming algorithm to calculate the concept similarity score between two terms, on top of the LCS dynamic programming algorithm for calculating word similarity. The two layers of dynamic programming not only result in a method less affected by missing words or words in different orders, but also significantly increase the query speed as we will see below. These enable our searching method practically applicable to many biomedical applications.

The UMLS Metathesaurus (version used in this work: 2012AB) contains around 11 million records in its MRCONSO.RRF files. Each record is a medical term. For query purposes, we discard duplicate terms and non-English terms and result in about 6.87 million records. A term is considered duplicate if both its CUI and name are identical to another term. However, among these 6.87 million records, there are only 1,874,573 unique words (white space is the delimiter). Thus concept similarity on a word basis saves a huge amount of redundant calculation otherwise needed by classic methods on a character basis. Correspondingly, in our method, we first pre-process the UMLS Metathesaurus into a word vector of unique words, and convert each UMLS concept, which consists of a list of words, into a list of indices with regard to the word vector. Procedure LDPMap-Preprocessing is the pseudo code.

Procedure LDPMap-Preprocessing ( )

1: **for** *i*=1: length (*Metathesaurus*)

2: *Word_Vector* = *Word_Vector* ∪ *Metathesaurus* [*i*];

3: **endfor**

4: **for** *i*=1: length (*Metathesaurus*)

5: **for** *j*=1: length (*Metathesaurus*[*i*])

WordIndex_vector [*i, j*] = the index of *Metathesaurus*[*i, j*] in *Word_Vector*;

6: **endfor**

7: **endfor**

8: **return** *Word_Vector*, *WordIndex_vector*;

We process a query using the Algorithm LDPMap_Query. When a query process starts, we first build a word similarity matrix between the query term and the word vector (Line 1-5), using the *WordSimilarity* function defined above. Then we build a concept score vector between the query term and 6.87 million UMLS Metathesaurus concepts (Line 6-8). The construction of the concept score vector uses the *WordSimilarityMatrix* built previously so that there are no more word similarity calculations. In addition, it adopts a dynamic programming approach in Function *ConceptSimilarityScore*, owing to the suboptimal structure of the *ConceptSimilarity* function.

Algorithm LDPMap_Query (*query_term*)

1: **for** *i*=1: length (*query_term*)

2: **for** *j*=1: length (*Word_Vector*)

3: *WordSimilarityMatrix*[*i, j*] = *WordSimilarity*(*query_term*[*i*], *Word_Vector*[*j*]);

4: **endfor**

5: **endfor**

6: **for** *i*=1:length(*Metathesaurus*)

7: *ConceptScore_Vector*[*i*] = *ConceptSimilarityScore*(*WordIndex_vector*[*i*]);

8: **endfor**

9: **return** Concepts in Metathesaurus corresponding to top scores in *ConceptScore_Vector*;

Function ConceptSimilarityScore (*WordIndex*)

1: **for** i=2:x+1

2: **for** j=2:y+1

3: $S(i, j) = WordSimilarityMatrix[i\text{-}1, WordIndex[j\text{-}1]]$;

4: **if** $S(i, j)+S(i\text{-}1, j\text{-}1) > max\ (S(i\text{-}1, j), S(i, j\text{-}1))$;

5: $S(i, j)= S(i, j)+S(i\text{-}1, j\text{-}1)$;

6: **else if** $S(i\text{-}1, j) > S(i, j\text{-}1)$

7: $S(i, j)=S(i\text{-}1, j)$;

8: **else**

9: $S(i, j)=S(i, j\text{-}1)$;

10: **endif**

11: **endfor**

12: **endfor**

13: **return** $2*S(x+1, y+1) / (x+y)$;

A Running Example

To facilitate the understanding of our method, we provide a simple running example of our method in Tables 1 and 2. Assume the input query term is "gastro reflux". The Algorithm LDPMap_Query will first build a WordSimilarityMatrix between this query term and the word vector of Metathesaurus. Results were partially shown in Table 1.

After the WordSimilarityMatrix is available, the Algorithm LDPMap_Query will calculate the concept similarity scores between the query term and UMLS concepts by dynamic programming. The calculation will refer to WordSimilarityMatrix for word similarity score instead of calculating it again. An example of a concept similarity calculation is given in Table 2.

Complexity Analysis

The LDPMap method is much faster than the classic LCS-based word similarity calculation by treating the query term and each UMLS concept as one single word, as demonstrated in our empirical study. The classic LCS-based word similarity calculation uses dynamic programming on a character basis while we use two layers of dynamic programming, one on a character basis and the other on a word basis. To understand the analytical reason behind this speedup, let us make some simple assumptions. Assume the UMLS Metathesaurus contains M unique concepts, and each concept or query term contains t words, and each word has d characters. Also assume UMLS Metathesaurus contains K unique words. Then, the classic LCS-based word similarity calculation takes approximately $O(t^2d^2M)$ time to handle a query. However, LDPMap method takes approximately $O(td^2K+t^2M)$ time to handle this query. It is easy to observe that $K<<tM$. This explains why LDPMap is much efficient. In the following, we will see that our LDPMap approach can be further sped up with the pipeline technique.

Speeding up LDPMap with the Pipeline Technique

In building the *WordSimilarityMatrix* and *ConceptScore_Vector*, the dynamic programming method has been used for around 1.87 million times and 6.87 million times, respectively. It is interesting to find out if there are repeated calculations that can be reused to speed up the LDPMap method. By studying both the word vector and

the Metathesaurus, we found the former has a lot of repeated prefixes among words (e.g. words “4-Aminophenol”, “4-Aminophenyl”), and the latter has a lot of repeated prefix words among concepts (e.g. C1931062 ectomycorrhizal fungal sp. AR-Ny3, C1931063 ectomycorrhizal fungal sp. AR-Ny2). Thus, by lexicographically sorting the word vector and the Metathesaurus, we can use this information to save a lot of calculation in the LDPMap approach as follows:

(1) In calculating *WordSimilarityMatrix*, Given a word *A*, if it has *p* common prefix letters with the previous word *B*, the dynamic programming only needs to start from *p*+1 iteration because the previous *p*+1 columns of the dynamic programming table are exactly the same as the previous results.

(2) In calculating *ConceptSimilarityScore*, Given a concept α, if it has *q* common prefix words with the previous concept β, the dynamic programming only needs to start from *q*+1 iteration because the previous *q*+1 columns of the dynamic programming table are exactly the same as the previous results. That means, the for loop in Line 2 of Function *ConceptSimilarityScore* shall start with $j=q+2$.

The mechanism of the speedup technique can be described as a pipeline technique because a computation result can be passed down and partially reused by the subsequent computation. In the empirical study, we will see that the pipeline technique significantly improves the LDPMap speed.

A Comprehensive Query Workflow Using LDPMap Approach

Given the above solutions to the concept similarity problem under Constraints 1 and 2, we will design a comprehensive query workflow for mapping a query term to UMLS concepts. Our query workflow needs to consider multiple types of input variations and errors. Other than missing words and words in different orders that can

be properly handled by concept similarity problem formulation, we need to consider another situation when two words are merged together. In this situation, the concept similarity modelling does not fit well because it is on a word basis. Therefore it is preferable to use the classic LCS method. However, as we pointed out above, the classic LCS method is too slow for the UMLS Metathesaurus. Fortunately, we found that we can leverage concept similarity solutions, outputting a list of words with similarity score great than a threshold. When we set the threshold to be 0.35, in most cases it is able to output concepts that are similar with the query term regardless of the word merging issues. The number of outputted concepts is much smaller than the size of UMLS Metathesaurus; thus applying the LCS method on this small subset is much faster than on the whole UMLS Metathesaurus. The query workflow is illustrated in Figure 1.

In the query workflow, we first calculate concept similarity scores under Constraint 2 between the query term and all UMLS concepts. If there are concepts with scores higher than threshold T_1, we output the results and the query completes. Otherwise, we save any concepts with scores higher than threshold T_2 as $SET(T_2)$, and then perform two additional queries: (1) calculate word similarity between the query term and each concept in $SET(T_2)$ by treating the query term and each concept as one single word; (2) calculate the concept similarity scores under Constraint 1 between the query term and all UMLS concepts. Finally, we merge and output the results from (1) and (2). The number of results outputted is adjustable. An application can choose to output concepts with scores higher than a threshold, or only the top ranked concepts.

Results

To understand the actual performance of LDPMap, we implemented it in C++, and subjected it to two sets of empirical studies. In summary, the results demonstrate that LDPMap method performs much better than available methods in terms of query speed and effectiveness. All experiments were carried out on Linux cluster nodes with 2.4GHz AMD Opteron processors. For the LDPMap query workflow, we set two parameters T_1=0.8 and T_2=0.35.

Query Speed Comparison

We would like to know how fast LDPMap handles query in comparison with the standard LCS method which treats the query term and each UMLS concept as a single word, and how effective the pipeline technique for the LDPMap is. Therefore, we test the three algorithms, LCS standard, LDPMap (LDPMap_Query Algorithm) without the pipeline technique, and LDPMap algorithm with the pipeline technique, on four sets of medical concepts randomly chosen from the UMLS Metathesaurus. The first set consists of 1000 single-word medical concepts. The second, third and fourth sets consist of 1000 two-word, 1000 three-word, and 1000 four-word concepts, respectively. The results are shown in Figure 2.

From Figure 2 we can observe that the LDPMap algorithm is much faster than the standard LCS. In addition, the standard LCS method is susceptible to the word numbers in a query term while the LDPMap method is much more stable. This result is consistent with the above complexity analysis. In addition, the LDPMap with the pipeline technique significantly speeds up the basic LDPMap method. This confirms our intuition that the pipeline technique saves huge amounts of redundant computation thus improving the efficiency of the LDPMap method. As a result, we can see that in this set of experiments LDPMap with pipeline techniques on average answers a query in less than 1 second. However, the standard LCS method takes about

1 to 2 minutes in answering a query, which makes it virtually unacceptable for many biomedical applications, which can require near real-time responses, or when processing large amounts of data. In addition to the slow query time, the standard LCS is not good at processing query terms with missing words or words in different orders, as we have discussed above.

It is worthwhile to note that even for one word query, LDPMap method is significantly faster than LCS, though the concept similarity is exactly the same as the word similarity in this case. This is because the LDPMap pre-processed the UMLS terms on a word basis and built an efficient index. The similarity measurement is not directly on the UMLS terms but on words and the index which saves a lot of computational cost. In contrast, the LCS will handle the similarity measurement directly over every UMLS term. This can also be explained by our complexity analysis above. When t=1 (t is the number of words in a query), LCS complexity is $O(d^2M)$ while the LDPMap is $O(d^2K+M)$. Since $K<<M$, we conclude that LDPMap is much faster than LCS.

Next, we would like to know how effective LDPMap handles queries, especially when the query terms are slightly different than the terms in the UMLS Metathesaurus.

Query Effectiveness Comparison

To understand how effective LDPMap (referring to LDPMap query workflow in this set of experiments) handles queries with name variations and errors, we used two available methods, UMLS Metathesaurus Browser and MetaMap as benchmarks. In a cursory examination of cTAKES, we found that it exhibited similar characteristics to MetaMap in its ability to handle name variations and errors and therefore we have excluded it from comparison. Since the study on UMLS Metathesaurus Browser

requires manually inputting terms and checking the results, we have to limit the query test to manageable numbers. In addition, since the UMLS Metathesaurus Browser cannot accept a query term with more than 75 characters, we limit all query terms in our test to be no more than 75 characters. Given the above situations, and considering the fact that more than 50% of UMLS concepts contain at least 32 characters, we randomly chose 100 medical concepts with 32-75 characters from the UMLS Metathesaurus.

The 100 medical concepts are divided into two groups. The first group consists of 50 concepts with no special characters (i.e., characters other than letters and numbers), and the second group contains 50 concepts with 5 or more special characters. The two groups are for two different testing purposes.

Group 1: We will use group 1 to test how effective the query workflow handles pure English name terms, and English name terms with input errors, variations, and typos. Thus, in addition to querying the original names, we also query the names with 1, 2, 3, and 4 character variations. Character variations are generated randomly in this study, including (1) deleting a character, (2) replacing a character, (3) merging two words, i.e., deleting the white space between two words.

Group 2: We will use group 2 to test how effective the query algorithm is in handling many professional medical terms, which may contain a good number of special characters, such as chemical compounds and drugs. To simulate the name variations that frequently appear in these terms, we randomly apply 1, 2, 3, and 4 character variations, including (1) deleting a special character, (2) replacing a special character by a white space.

To complement the above test groups, we use the following group to test how effective the query algorithm handles short terms which may be queried commonly in real situation.

Group 3: We randomly picked 100 medical concepts with 5-31 characters. Since many of these concepts are quite short, we only apply 1 and 2 random character variations, including (1) deleting a character, (2) replacing a character, (3) merging two words.

In these experiments, we found that MetaMap often output multiple matching results but there are no ranks of these results. In contrast, the UMLS Metathesaurus Browser usually outputs a list of ranked concepts, and LDPMap can be configured to output the top k (k>=1) ranked concepts.

Thus, to be as fair as possible, we use two criteria to measure the correctness of a query:

Criterion 1: A query is correct if the original term appear (1) in top 25 ranked concepts (i.e., in the first page of the result) by the UMLS Metathesaurus Browser; (2) in the top 25 ranked concepts by LDPMap; (3) in the result of MetaMap.

Criterion 2: A query is correct if the original term appears (1) as the top ranked concept by UMLS Metathesaurus Browser; (2) as the top ranked concept by LDPMap.

Criterion 1 indicates if the query processing mechanism is able to handle the query with reasonable accuracy. Criterion 2 is much stringent and it indicates whether a method can be applied to applications require high accuracy.

Figures 3 and 4 are the error rate for the two groups of experiments, under Criterion 1. From both figures, we can clearly see that the LDPMap approach has very few errors among all tests. In comparison, the UMLS Metathesaurus Browser and MetaMap's

error rate are quite high especially when multiple characters changes are present. MetaMap has a considerable error rate even when querying the original terms (0 characters changes). This may owe to the text processing mechanism of MetaMap. Since MetaMap is targeted at finding medical terms from a biomedical text, it leverages a combination of part-of-speech tagging, shallow parsing, and longest spanning match against terms from the SPECIALIST Lexicon before matching terms against concepts in the UMLS. Therefore, it tends to decompose longer spans of text and medical terms into several shorter medical terms.

Figures 5 and 6 are the error rates for the two groups of experiments, under Criterion 2. Since MetaMap usually outputs multiple concepts without ranking, we exclude MetaMap from the Criterion 2 measurement. From these two figures, we can observe that the error rate of the UMLS Metathesaurus Browser is much higher in comparison with the measurement of Criterion 1. Quite surprisingly, there are some errors even when querying a few original terms (such as " Distal radioulnar joint"). This suggests that UMLS Metathesaurus Browser is not suitable for query processing for applications that have a high-accuracy demand. In contrast, the LDPMap still has a very low error rate, on average less than 5% across the 0-5 character changes, and free of errors in querying the original terms.

From Figures 7 and 8, we can see that the general performances of LDPMap, UMLS Metathesaurus Browser, and MetaMap on short query terms are similar to their performances on long query terms. LDPMap still has a clear advantage over UMLS Metathesaurus Browser, and MetaMap. However, we noticed that LDPMap error rate reaches 27% for 2 character changes under Criterion 2. This is understandable because generally short terms contain fewer words than long terms, and the concept similarity measurement is less favoured. However, the parameter T_1 can be used as an

adjustment of preference between the concept similarity measurement and the word similarity measurement. By increasing T_1 from 0.8 to 0.85, we observed that this error rate reduces from 27% to 20%. This demonstrates that LDPMap is flexible in handling both long and short term queries.

To provide some details on the medical concepts we used in this set of experiments, and the character changes applied. We list a few of them in Tables 3. From this table, we can see that it contains concepts of different lengths. The randomly generated character variations cover several common cases of text data inaccuracy, including, misspellings, merging of two words, and special character omissions. From Table 4 we can see that MetaMap cannot handle them properly. Instead, it finds some concepts related to individual words in the query term. The UMLS Metathesaurus Browser does not do any better on them. In contrast, LDPMap correctly answered all these queries except for "AlbunexIectable Product". Although "Injectable Product" is not correct, it is at least closer to the original term than those returned by the UMLS Metathesaurus Browser and MetaMap. By reviewing the LDPMap approach, we conclude that this error can be eliminated if we increase the threshold T_1 to a value such that word similarity (LCS) is used to measure the two terms. To confirm this, we increase T_1 from 0.8 to 0.85, and LDPMap successfully returns the original term. However, a high T_1 implies that LDPMap gives more preference to LCS-based similarity measurement than to concept similarity measurement defined above. Consequently, LDPMap will be less productive in handling real-world queries that contain incomplete medical terms (i.e., medical terms with missing words). It is quite evident that there does not exist one set of T_1 and T_2 that fits all situations. As a result, we will fine tune these parameters to leverage LDPMap in our future applications.

Conclusions

In the work we proposed LDPMap, a layered dynamic programming approach to efficiently mapping inaccurate medical terms to UMLS concepts. As a main advantage of the LDPMap algorithm, it runs much faster than classical LCS method therefore makes it possible to efficiently handle UMLS term queries. When similarity is counted on a word basis, LDPMap algorithm may yield a more desirable result than LCS. In other cases (such as word merging), it is possible that LCS query results are more preferable. Thus, in the comprehensive query workflow of LDPMap, the LDPMap method is complemented by LCS and adjustable by parameter T_1. Different from using LCS alone, the LDPMap query workflow only applies LCS (when needed) to a very limited number of candidate terms thus achieves a very fast query speed.

In query effectiveness comparison, we observed that LDPMap has a very high accuracy in processing queries over the UMLS Metathesaurus involving inaccurate terms. In contrast, the UMLS Metathesaurus Browser has a very limited ability in handling these queries, though it can handle queries of accurate terms fairly well. Throughout the study, we also observed that MetaMap, in general, is not suitable for mapping long medical terms to the UMLS concepts as it focuses on extracting short medical terms from the query text.

Although LDPMap is very efficiently in handling UMLS term queries, it has two major limitations. First, it cannot handle synonyms and coreferences. Fortunately, UMLS Metathesaurus often list a concept preferred names and synonyms so that LDPMap can work effectively in most cases, though the list may still not be complete. Second, it is not able to perform syntax-level processing as MetaMap does, such as extracting medical terms from an article. Whether it is possible to extend the LDPMap approach to overcome the two limitations remains an open question. In the future we would like to investigate this question and plan to use LDPMap as an efficient pre-

processing tool to map medical terms to the UMLS concepts, and use the results in our knowledge discovery platform.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

KR implemented the LDPMap algorithm, carried out the experiments, and edited the manuscript. AL, AM, RM, and KH analyzed comparable methods, participated in the design of the study, and revised the manuscripts. YX led the project including development of the idea, design of the algorithms, and writing of the manuscript.

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Figures

Figure 2

Query time of LCS, LDPMap and LDPMap pipeline on randomly chosen 1000 medical concepts.

Figure 3

Correctness comparison on LDPMap, UMLS Metathesaurus Browser, and MetaMap for Group 1 using Criterion 1.

Figure 4

Correctness comparison on LDPMap, UMLS Metathesaurus Browser, and MetaMap for Group 2 using Criterion 1.

Figure 5

Correctness comparison on LDPMap and UMLS Metathesaurus Browser for Group 1 using Criterion 2.

Figure 6

Correctness comparison on LDPMap and UMLS Metathesaurus Browser for Group 2 using Criterion 2.

Figure 7

Correctness comparison on LDPMap, UMLS Metathesaurus Browser, and MetaMap for Group 3 using Criterion 1.

Figure 8

Correctness comparison on LDPMap and UMLS Metathesaurus Browser for Group 3 using Criterion 2.

Tables

Table 1.

An example of *WordSimilarityMatrix* constructed for query term "gastro reflux".

		***Word_Vector* of Metathesaurus**						
		…	gastro (at i)	…	Oesophageal (at j)	…	reflux (at k)	…
Query term	gastro	…	1 (gastro)	…	0.235294 (so/ga)	…	0.166667(r)	…
	reflux	…	0.166667 (r)	…	0.235294 (el)	…	1(reflux)	…

Table 2

An example of calculating the concept similarity score between the query term "gastro reflux" and the UMLS concept "gastro oesophageal reflux" for the *ConceptScore_Vector* construction. The calculation will refer to the *WordSimilarityMatrix* as shown in Table 1. The normalized final similarity score is 2*2/(2+3)=0.8.

		UMLS concept	gastro	oesophageal	reflux
		word index	i	k	j
query term	order		0	0	0
gastro	1	0	1	1	1
reflux	2	0	1	1.23594	2

Table 3

Original terms and their randomly generated character variations

CUI	name	Randomly generated 4 character variations
C3267394	POMEGRANATE FRUIT EXTRACT 150 MG Oral Capsule	POMGRAATE FRUIT EXTRdCT 150 MG Oral Casule
C3228202	Albunex Injectable Product	AlbunexIectable Product
C0505183	Lateral branch of dorsal ramus of fifth thoracic spinal nerve	LateMa branch of dorsal ramus of ifth thoracic gpinal nerve
C1459293	Sinorhizobium americanus	Sinokhizrbimamericanus
C1541607	gp100/IL-7/ISA-51/MART-1	gp100 IL 7ISA-51/MART1
C1352046	danthron 1.5 MG/ML / Pantothenic Acid 2.5 MG/ML Oral Suspension	danthron 15 MGML Pantothenic Acid 25 MG/ML Oral Suspension
C0040372	Benzenesulfonamide, N-(((hexahydro-1H-azepin-1-yl)amino)carbonyl)-4-methyl-	Benzenesulfonamide, N-((hexahydro1H-azepin-1-yl amino)carbonyl-4-methyl-
C2714409	1-undecene-1-O-beta-2',3',4',6'-tetraacetyl glucopyranoside	1-undecene1-O-beta2,3',4',6-tetraacetyl glucopyranoside

Table 4

Query results for Table 3.

CUI	UMLS Metathesaurus Browser (concept ranked 1st by approximate match)	MetaMap	LDPMap
C3267394	C0030054 Oxygen	C0016767 Fruit, C2346927 Mg++, and 4 others	correct
C3228202	C1514468 product	C1704444 Product (Multiplicative Product) [Quantitative Concept] C1514468 product [Entity]	C0086466 Injectable Product
C0505183	C0007965 Chediak-Higashi Syndrome	C1706131 Branch(Branch(group)), C2700383 Branch(Branch of plant), and 6 others	correct
C1459293	No result	No result	correct
C1541607	C1512807 Integrated Learning System	C0020898 IL (Illinois (geographic location)), C1522481 MART-1 (MART-1 Tumor Antigen) , and 2 others	correct
C1352046	C0029383 Osmium	C1129294 danthron 25 MG, C0439526 /mL [Quantitative Concept], and 3 others	correct
C0040372	C0265215 Meckel-Gruber syndrome	C0053169 benzenesulfonamide, C0441922 N+ (N+ (tumor staging)), and two others	correct
C2714409	C0030011 Oxidation	C0470206 +1 [Q uantitative Concept] C1417683 BETA2 (NEUROD1 gene), and 7 others	correct